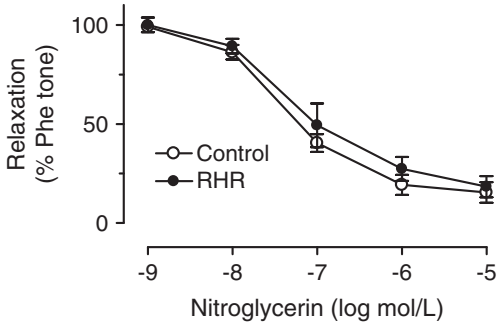
# Supplementary Data

## Supplementary Methods

### *Vascular reactivity*

The interlobar renal arteries (mean diameter of ~250 $\mu$m) were dissected in ice-cold Krebs solution ([m*M*]: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 $KH_2PO_4$, and 11 D-glucose) oxygenated by 95% $O_2$–5% $CO_2$ at 37°C (pH ~7.4) and suspended in a myograph (Danish Myo Technology) to record changes in isometric tension (3). In some arteries, the endothelium was mechanically removed by rubbing the inner surface of the segment with a fine steel wire, and functional removal of the endothelium was confirmed by the absence of relaxation in response to acetylcholine (10 $\mu M$). The changes in endothelium-dependent relaxations (EDRs) induced by cumulative concentration (0.01–30 $\mu M$) of acetylcholine were determined in arteries contracted by phenylephrine (1 $\mu M$). SC-560 (0.3 $\mu M$; cyclooxygenase [COX]-1 inhibitor), valeryl salicylate (VAS, 300 $\mu M$; COX-1 inhibitor); celecoxib, NS398, DuP697 (3 $\mu M$; COX-2 inhibitor), indomethacin (1 $\mu M$; non-selective COX inhibitor); tiron (1 m*M*; superoxide dismutase mimetic) plus diethyldithiocarbamate acid (DETCA, 100 $\mu M$; hydroxyl radical [$HO^{\bullet}$] scavenger], tempol (100 $\mu M$), apocynin (100 $\mu M$, NADPH oxidase inhibitor), allopurinol (100 $\mu M$ xanthine oxidase inhibitor), S18886 (0.1 $\mu M$; thromboxane receptor antagonist), and cycloheximide (10 $\mu M$, protein synthesis inhibitor) were added 30 min before EDRs were performed. Endothelium-independent relaxations to sodium nitroprusside or nitroglycerin were studied in arteries without endothelium. To examine endothelium-dependent contractions (EDCs), rings were first treated for 30 min with 100 $\mu M$ $N^G$-nitro-L-arginine methyl ester (L-NAME) to eliminate the interference of endothelium-derived nitric oxide (NO) (5), and then contractions were elicited by acetylcholine (0.1–100 $\mu M$). The effects of all COX inhibitors including NS-398, celecoxib, DuP-697, VAS, SC-560, indomethacin, S18886, cycloheximide, tiron plus DETCA, allopurinol, or tempol were examined on EDCs using the same concentration as in EDR experiments. The role of reactive oxygen species (ROS) induced contraction in rings from renovascular hypertensive rat (RHRs) and control rats were examined by using hydrogen peroxide ($H_2O_2$, 100 $\mu M$) or a combination of hypoxanthine (HX, 100 $\mu M$) and xanthine oxidase (XO, 0.01 unit/ml) to induce contractions in the presence of 100 $\mu M$ L-NAME. Prostaglandins including prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 8-isoprostane, and prostacyclin ($PGI_2$) were also used to induce contractions in renal arteries from RHRs and control rats. The effect of $PGF_{2\alpha}$ (7 n*M*, a concentration that did not cause contraction) on EDRs in renal arteries of control rats was studied with or without the presence of S18886 or celecoxib.




**SUPPLEMENTARY FIG. S1. Endothelium-independent relaxations were not altered in renal arteries of RHRs.** Nitroglycerin-induced endothelium-independent relaxations were similar between control and renovascular hypertensive rat (RHR) groups.

### *Measurement of prostaglandins by enzyme immunoassay*

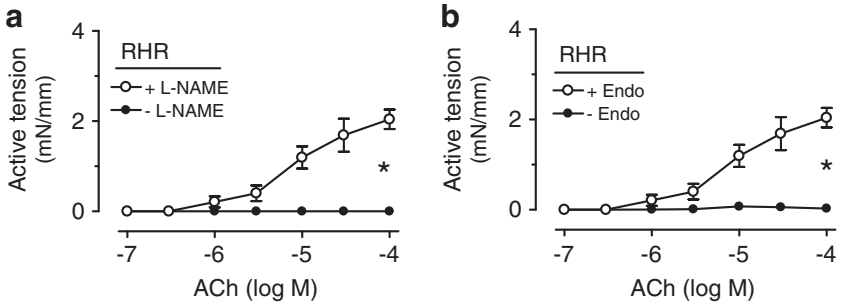
The amounts of individual prostaglandins were measured by enzyme immunoassay kits (Cayman Chemical). Rat renal arteries were exposed for 5 min to acetylcholine (100 $\mu M$) in a 500-$\mu$l Krebs solution with and without acute inhibitor treatment; the solution was collected and kept at −80°C. Arterial tissues were removed, and their protein concentrations were determined using the Lowry method. Six prostaglandins or their metabolites, namely $PGF_{2\alpha}$, $PGE_2$, $PGD_2$, 6-keto $PGF_{1\alpha}$ (for $PGI_2$), $TXB_2$ (for $TXA_2$), and 8-isoprostane, were assayed.

### *Measurement of renal blood flow by magnetic resonance image acquisition procedure*

Magnetic resonance image (MRI) studies were performed using a 3 T clinical whole-body imaging system (Achieva; Philips Healthcare) as described previously (2). After anesthesia, rats were positioned supine. The MRI acquisition of the rat urinary system included high-resolution T2 weighted axial plane anatomical examination; high-resolution T1 weighted coronal plane anatomical examination; and dynamic contrast-enhanced examination in coronal plane. Axial anatomical examinations were acquired with the following parameters: multiple slice turbo spine echo sequence, repetition time (TR)/time to echo (TE)/flip angle = 2359 ms/120 ms/90°, field of view = 60 × 81 × 30 mm, the acquisition voxel size was 0.41 × 0.41 × 1.50 mm, and the reconstructed voxel size was 0.17 × 0.17 × 1.5 mm. Coronal anatomical examinations were acquired with the following parameters: three-dimensional (3D) gradient echo sequence with fat suppression, TR/TE/flip angle = 4.4 ms/2.2 ms/10°, field of view = 80 × 80 × 18 mm, the acquisition voxel size was 0.50 × 0.50 × 1.00 mm, and the reconstructed voxel size was 0.28 × 0.28 × 0.50 mm. The contrast-enhanced examinations were acquired with the following parameters: 3D gradient echo sequence, TR/TE/flip angle = 6.8 ms/2.3 ms/35°, field of view = 80 × 80 × 12 mm, the acquisition voxel size was 0.61 × 0.75 × 3.00 mm, and the reconstructed voxel size was 0.31 × 0.31 × 1.5 mm. The temporal resolution was two seconds per acquisition. MRI contrast agent was gadolinium-tetraazacyclododecanetetraacetic acid (Guerbet Group; Roissy CDG cedex). A dose of 0.075 mmol/kg was injected through the tail vein as a rapid bolus in less than 1 s after initial baseline 10 acquisitions and followed by a flush of 0.5 ml normal saline. Dynamic scan was stopped when the contrast agent was excreted and clearly visible in the bilateral ureters.

### *MRI analysis*

The reconstructed MR images were transferred to a radiological workstation (Extended Workspace; Philips) for off-line

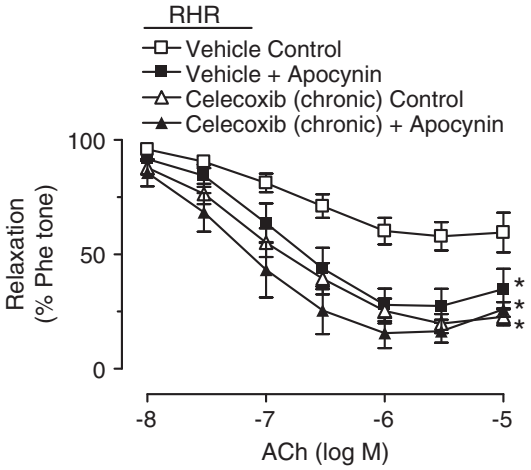



**SUPPLEMENTARY FIG. S2. Endothelium-dependent contractions in RHR renal arteries were seen upon blockage of the NO synthesis.** Acetylcholine (Ach)-elicited contractions occurred in renal arteries of the RHRs only in the presence of 100 $\mu M$ $N^G$-nitro-L-arginine methyl ester (L-NAME) **(a)** and with endothelium **(b)**. Results are means ± standard error of the mean (SEM) of six experiments. *$p < 0.05$ *versus* + L-NAME and + Endo.

analysis. Anatomical images were read by a radiologist with small animal research experience. For analysis of dynamic data, regions of interest (ROIs) were manually drawn over left and right kidneys. The ROIs of the kidney included the cortex only, and consistent ROIs were drawn in all rats. These ROIs were used on the dynamic scan data to generate time signal intensity curves.

### *Electron paramagnetic resonance spectroscopy*

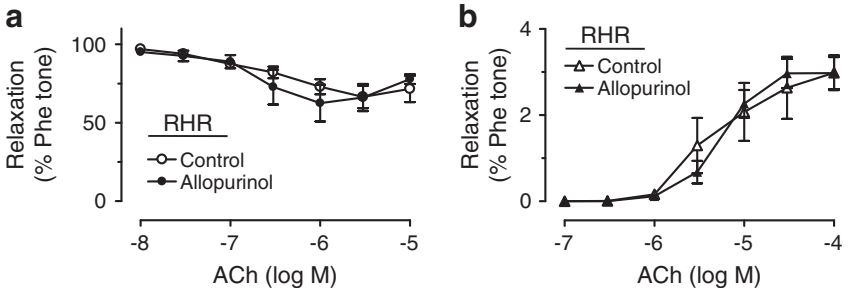
The formation of ROS was measured by a reaction with the spin trap agent TEMPONE-H using electron paramagnetic resonance (EPR) spectroscopy, the method adopted from Dikalov *et al.* (1). Diethylene-triaminepentaacetic acid (DTPA) was used to decrease the self-oxidation of hydroxylamine catalyzed by traces of transition metal ions. Renal arteries were dissected from RHRs or control rats, and incubated in DTPA (100 $\mu M$) and TEMPONE-H (80 $\mu M$) at 37°C in oxygenated Krebs solution for 30 min. Acetylcholine was added for 5 min to trigger the release of ROS in the presence of L-NAME (100 $\mu M$). Tissues together with 200 $\mu$l incubation medium were collected, homogenized, and stored in glass Pasteur pipettes (tip inner diameter: 1 mm) at −80°C. The samples were thawed at room temperature before the start of the measurement. EPR spectra were recorded at room temperature using a Bruker EMX EPR spectrometer (Bruker). The EPR settings were as follows: field center, 3475 G; field sweep, 60 G; microwave frequency, 9.72 GHz; microwave power, 20 mW; magnetic field modulation, 100 kHz; modulation amplitude, 2.0 G; conversion time, 655 ms; detector time constant, 1024 ms; and acquisition of ten scans. The generation of ROS by chemical interaction between xanthine oxidase (XO, 0.01 unit/ml) and hypoxanthine (HX, 100 $\mu M$) was taken as the positive control (4). A mixture of Krebs solution containing DTPA and TEMPONE-H served as the negative control. TEMPONE-H was dissolved in double-distilled water.



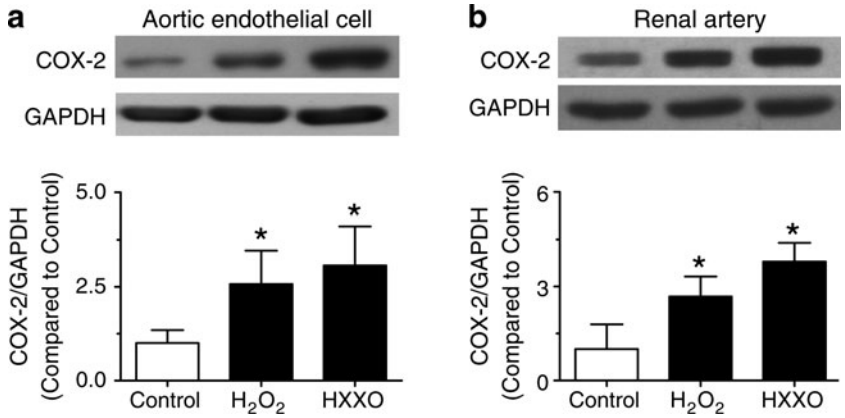

**SUPPLEMENTARY FIG. S3. Role of NADPH oxidases.** Acute incubation of 100 $\mu M$ apocynin for 30 min improved endothelium-dependent relaxations (EDRs) in renal arteries from RHR vehicle, whereas it did not affect EDRs in renal arteries from RHRs after celecoxib chronic treatment. Results are means ± SEM of six to eight experiments from different rats. *$p < 0.05$ *versus* vehicle control.

### *Western blotting*

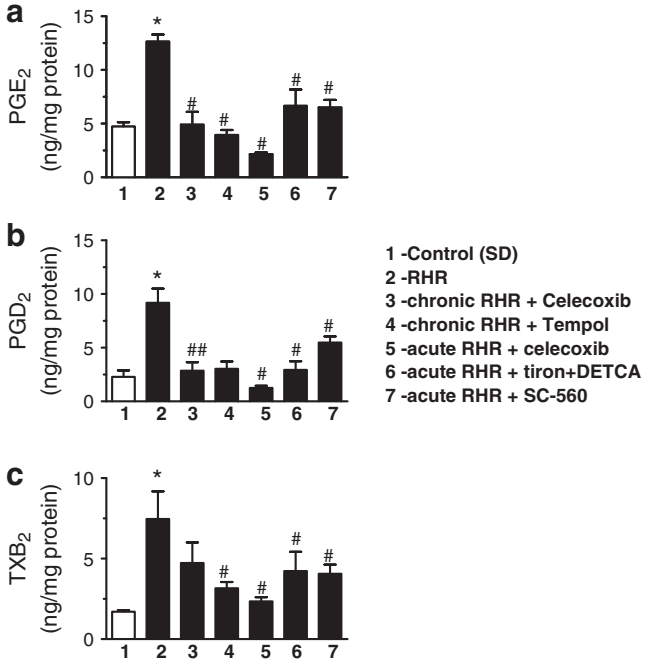
Renal arteries were dissected out, homogenized at 4°C in lysis buffer (10 m$M$ Tris-HCl, 150 m$M$ NaCl, 1 m$M$ EDTA, 25 m$M$ sodium pyrophosphate, 1 m$M$ $\beta$-glyco-phosphate, 1 m$M$ sodium orthovanadate, 2.1 $\mu M$ leupeptin, 1 mg/ml aprotinin, 1 m$M$ phenyl-methylsulfonyl fluoride, and 1% Triton X-100), and incubated on ice for 10 min. Samples were then centrifuged at 20000 $g$ for 20 min at 4°C, and the supernatant was collected. Protein concentrations were determined using the Lowry method. The protein samples were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto an immobilon-P polyvinylidene difluoride membrane (Millipore). Nonspecific binding sites were blocked by 5% nonfat milk in 0.05% Tween-20 phosphate-buffered saline, and then incubated overnight at 4°C with primary antibodies including COX-2 or COX-1 (Cayman); and 3-nitrotyrosine (1:1000; Upstate Biotechnology); BMP4 (1:1000; Sigma), phospho- and total p38MAPK, $p67^{phox}$ (1:1000; Cell Signaling), and NOX2 (1:1000; Abcam). The blots were incubated with secondary antibodies (DakoCytomation) for 1 h at room temperature. The membranes were then developed with an enhanced chemiluminescence detection system (ECL reagents; Amersham Pharmacia), and finally exposed to X-ray films. Equal protein loading was verified with the help of a housekeeping anti-GAPDH antibody, and the GAPDH level was used for the normalization of expression of the other proteins.



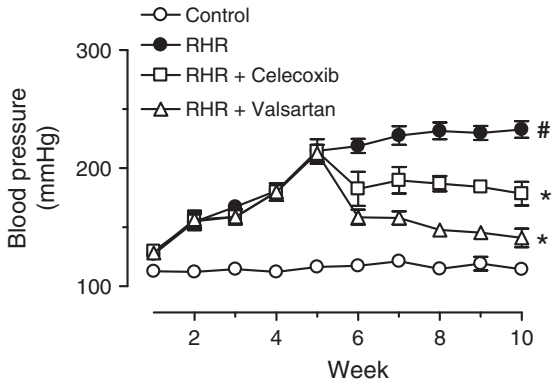

**SUPPLEMENTARY FIG. S4. Role of xanthine oxidase.** Acute incubation of 100 $\mu M$ allopurinol for 30 min did not change EDRs **(a)** or EDCs **(b)** in renal arteries from RHR. Results are means ± SEM of four experiments from different rats.




**SUPPLEMENTARY FIG. S5. COX-2 up-regulation upon ROS stimulation.** $H_2O_2$, (100 $\mu M$), or a combination of HX (100 $\mu M$) and XO (0.01 unit/ml) increased cyclooxygenase (COX)-2 expression in primary rat aortic endothelial cells **(a)** and renal arteries **(b)** from control rats after 4 h of incubation. Results are means ± SEM of six from different rats. *$p < 0.05$ *versus* control.




**SUPPLEMENTARY FIG. S6. Prostaglandin synthesis.** Ach (100 $\mu M$) stimulated release of prostaglandins **(a)** $PGE_2$, **(b)** $PGD_2$ and **(c)** $TXB_2$ in renal arteries from the RHRs and the control rats in response to drug treatments. Results are means ± SEM of four experiments. *$p < 0.05$ *versus* control and $^{\#}p < 0.05$ *versus* RHR.




**SUPPLEMENTARY FIG. S7. Blood pressure.** Blood pressure measurement weekly after the induction of renal artery stenosis of four groups of rats including control, RHR, RHR orally treated with celecoxib (10 mg/kg/day), and RHR treated with angiotensin receptor blocker valsartan (10 mg/kg/day). Valsartan was used as a positive control to compare with celecoxib on blood pressure. Oral administrations of both drugs started 5 weeks after surgery, which lasted for another 5 weeks. Results are means ± SEM of six experiments. $^{\#}p < 0.05$ *versus* control. *$p < 0.05$ *versus* RHR.

## References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