Supplemental Material
Supplemental Methods

Animal experiments

Effects of oral nitrate supplementation on arterial pressure

At the age of ten weeks, six rats per group were implanted with radiotelemetric devices for arterial pressure and heart rate recordings (TA11PA-C40 or HD-S10, DSI-Transoma Medical, St Paul, MN) as described previously 1. After one week of recovery, animals were transferred into metabolism cages. Each treatment group contained two additional rats without telemetric devices in order to have sufficient sample size for chemical and molecular analyses. Receivers for telemetry signals were placed behind the metabolism cages for simultaneous arterial pressure recordings and 24 h urine collections. The animals were allowed to adapt for two days to the new environment.

Then, animals were randomized to four treatment protocols with the substances administered via a purified diet (EF R/M, sodium deficient ssniff, Soest, Germany). Sodium chloride (final content 0.6%), distilled water, sunitinib and sodium nitrate were added to the food in amounts to achieve the respective dosages. Depending on the body weight, the animals received 30-40 g of this diet per day which was taken up completely. Group 1 received a control diet for six days; group 2 received a diet supplemented with sodium nitrate resulting in a nitrate uptake of 1 mmol/(kg *d) 2 for six days; group 3 received a control diet for two days followed by a sunitinib-containing diet [15 mg/(kg*d)] for four days; group 4 received a nitrate-supplemented diet for two days followed by a nitrate-supplemented and sunitinib-containing diet for four days. 24h urine collections were performed as previously described 1,3. At the end of the experiments, animals were deeply anesthetized with pentobarbital and the kidneys were removed, blotted and dissected into cortex and medulla on ice cooled petri dishes, snap frozen in liquid nitrogen and stored at -70°C for later analyses.

Effects of NO synthase (NOS) inhibition on renal function
Rats received sunitinib (sunitinib malate, LC Laboratories, Woburn, MA, USA) at 15 mg/(kg*d) or vehicle for four days as described above. On day five, animals were anesthetized with pentobarbital (60 mg/kg, i.p.) and instrumented with a carotid artery catheter for arterial pressure recordings and blood sampling and a jugular vein catheter for intravenous infusion of isotonic saline containing 4 mg/ml inulin and 10 mg/ml bovine serum albumin. The infusion rate was 1.2 ml/(h*100 g body weight). Rats were further instrumented with a tracheal cannula for artificial ventilation and a femoral vein catheter for administration of pentobarbital [20 mg/(kg*h)], pancuronium bromide [1 mg/(kg*h)] and L-NAME. Urine was sampled via ureter catheters and renal blood flow (RBF) was recorded with an ultrasound transit time flow-meter (Transonic Systems, Ithaca, NY) placed around the left renal artery. A fine polyethylene catheter was inserted into the left femoral artery and its bent tip advanced to the branching point of the left renal artery from the aorta. This catheter served for intrarenal drug administration and was continuously perfused with isotonic saline at 3 µl/min, when no drugs were given. Exact catheter placement was verified by a fall in RBF in response to acetylcholine (17 ng, i.a.) without effects on systemic arterial pressure.

For experiments involving intrarenal artery L-NAME administration animals were additionally instrumented with a Laser-Doppler flow probe which was advanced into the renal medulla of the left kidney with the aid of a stereotaxic apparatus (David Kopf, Instruments, Tujunga, CA) and coupled to a Laser-Doppler flowmeter (Perimed, Järfälla, Sweden). To prevent movement artifacts, the left kidney was placed in a plastic kidney holder and covered with gauze soaked with isotonic saline. Urine flow was measured gravimetrically. At the end of each clearance period, 500 µl of arterial blood were sampled. Sodium and inulin concentrations were measured in plasma and urine and the data were used to calculate glomerular filtration rate (GFR) and fractional sodium excretion FE\textsubscript{Na}. Acetylcholine, N\textomega-nitro-L-arginine methyl ester (L-NAME) and inulin were from Sigma Aldrich, Munich, Germany.

**Experiment 1 (intravenous L-NAME):** After a 45 min postsurgical equilibration period, a dose-response curve on the effect of acetylcholine on renal vascular resistance (RVR) was obtained. Acetylcholine was administered via the renal artery at increasing doses that caused minimal effects on systemic arterial pressure (blood pressure reductions less than 10 mmHg). Depending on the dose, acetylcholine administration lasted between 2.5 and 10s which resulted in a sudden rise in RBF. Thereafter, GFR
and \( FE_{Na} \) were measured over a 30 min clearance period. This period was followed by intravenous administration of the non-selective NOS inhibitor L-NAME at 10 mg/kg \(^4\). After another 30 min, the protocol was repeated (Fig. S1). At the end of the experiments, the animals were killed with an overdose of pentobarbital and the kidneys were removed, blotted and weighed.

**Experiment 2 (intrarenal artery L-NAME):** To test whether NO differentially contributes to the regulation of \( FE_{Na} \) and renal medullary blood flow (RMF) in control and sunitinib-treated animals, baseline data on renal hemodynamics and \( FE_{Na} \) were obtained during a 30 min clearance period that was started after 45 min of postsurgical equilibration. Thereafter, L-NAME was administered at 250 \( \mu \)g/kg via the renal artery catheter (infusion rate: 5 \( \mu \)g L-NAME/min). We opted for this L-NAME dose and this route of administration to selectively inhibit renal NOS with minimal systemic effects that could counteract the effects of NOS inhibition on \( FE_{Na} \) \(^5\), \(^6\). Thereafter, renal hemodynamic parameters and \( FE_{Na} \) were measured for another 30 min (Fig. S1). At the end of the experiments, animals were killed with an overdose of pentobarbital and the kidneys were removed, blotted and weighed. Side-specific NOS inhibition was verified by renal function parameters that were obtained simultaneously from both kidneys during L-NAME infusion into the left renal artery (Fig. S2).

**Blood pressure response to cinaciguat**

At the age of ten weeks, rats were implanted with radiotelemetric devices (HD-S10, DSI-Transoma Medical, St Paul, MN). After one week of recovery, arterial pressure and heart rate recordings were started. After two days of recording baseline data, animals were placed on a sunitinib-containing diet [15 mg/(kg*d)] for four days and randomized to two protocols. **Group I** continued to receive sunitinib [15 mg/(kg*d)] only, whereas **group II** received the soluble guanylate cyclase (sGC) activator cinaciguat [BAY 58-2667, 10 mg/(kg*d) for one day] in addition to sunitinib with the diet. Cinaciguat was kindly provided by Bayer AG Pharmaceuticals Division, Leverkusen, Germany. Pilot studies conducted prior to this experiment had revealed that the combined administration of sunitinib and cinaciguat for more than one day was not well tolerated by the animals leading to decreased food and hence drug intake. The effects
on food intake were already evident after 24h, when ingestion was reduced by 20-30 per cent. To control for these effects, we opted for a pair feeding design with rats in group I receiving the same amount of food that had been ingested by the rats in group II.

Chemical analyses

Plasma and urine samples were analyzed for inulin and Na\(^+\) concentrations as described previously ¹ ³. GFR and FE\(_{Na}\) were calculated according to standard formulas. Urinary nitrite concentrations were determined by diazo dye formation and urinary and plasma nitrate concentrations were determined enzymatically via the nitrate reductase reaction with a test kit from R-Biopharm, Darmstadt, Germany following the manufacturer’s instructions. Diazo dye and NADPH concentrations were measured spectrophotometrically. Nitrite and nitrate concentrations (NO\(_x\)) were added and 24h renal NO\(_x\) excretion was calculated from NO\(_x\) concentration and 24h urine volume. The detection limit of the nitrate reductase reaction for nitrate was 2 µmol/l. Urinary cGMP concentrations were measured using a competitive ELISA with acetylcholine esterase-conjugated cGMP as tracer (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions. Urine samples were collected in metabolism cages and kept frozen at -20°C until analyzed. Samples were diluted at 1:1000 for analyses. Twenty-four-hour cGMP excretion rates were calculated from the cGMP urine concentrations and urinary excretion rates and normalized to 100 g body weight.

Renal mRNA and protein abundances

Tissues were obtained from rats treated with sunitinib at 15 mg/(kg*d) or vehicle for four days. Kidneys were dissected into cortex and medulla. Samples were frozen in liquid nitrogen and stored at -70°C until further analyses.

mRNA: For NOS mRNA determination, tissues were obtained from sunitinib-treated animals and controls that had not been subjected to invasive experiments. Tissue samples of 70-80 mg were homogenized in Trizol (peqGOLDTrifast, PEQLAB Biotechnologie, Erlangen, Germany) and centrifuged. Chloroform was added to the
supernatants to separate RNA from accompanying organic material into the aqueous phase by centrifugation. This was followed by DNase I treatment of the RNA isolate to remove residual genomic DNA and subsequent RNA concentration by ammonium acetate precipitation (RNAqueous, Ambion, Austin, TX). For sGC mRNA determination, tissue samples were obtained from the two groups of the nitrate supplementation experiment that did not receive nitrate. 25 mg tissue were homogenized in Trifast and centrifuged. Ethanol was added to the supernatant and samples were further processed according to the manual of Direct-zol RNA MiniPrep (Zymo Research Europe, Freiburg, Germany). DNase I treatment was carried out in-column.

cDNA was reverse transcribed using 1 µg RNA per reaction and random hexamer primers (High-Capacity cDNA Reverse transcription Kit, Applied Biosystems, Foster City, CA). cDNA abundances were analyzed by qPCR with a SYBR Green PCR kit (Rotor-GeneSYBR Green; Qiagen, Venlo, The Netherlands) and a real-time PCR cycler Rotor Gene Q (Qiagen, Hilden, Germany). Oligonucleotide primers for NOS and Ywhaz (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) were obtained from Invitrogen, Wesel, Germany; sequences are given in Table 1. sGC primers were obtained as QuantiTect Primer Assays from Qiagen (Assay Name: Rn_Gucy1a3_1_SG [GenBank accession number NM_017090]; Rn_Gucy1b3_1_SG [GenBank accession number NM_012769]). Sequence data are not provided by this supplier. Relative cDNA abundances were determined by the ∆∆Ct method. ∆Ct values were calculated for the housekeeping gene Ywhaz and the NOS isoenzymes or the sGC subunits. Then, the mean value of control group ∆Ct values was calculated. This mean value was used to calculate the ∆∆Ct values between control and treatment groups.

Protein isolation for determination of sGC abundance: Frozen powdered renal tissue was homogenized in 10 volumes of ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v) and 1x protease inhibitor cocktail (Sigma-Aldrich). Samples were treated twice with supersonic waves for 15 s at 4°C and centrifuged at 10,000 g for 15 min at 4°C. Supernatant protein concentration was determined using the BCA kit (Pierce BCA Protein Assay Reagent Kit, Pierce).
**sGC Western blot:** Equal amounts of protein (25 µg) were electrophoretically separated on a 4-15% sodium dodecyl sulfate (SDS) polyacrylamide gel (Bio-Rad, Munich, Germany) and transferred to polyvinylidene fluoride membranes (Carl Roth, Karlsruhe, Germany). The membranes were blocked with nonfat dry milk and probed with an anti-sGC antibody followed by incubation with peroxidase-labeled secondary antibody (1:2,000, rabbit anti-goat, Merck Millipore, Darmstadt, Germany). The following anti-sGC antibodies from Abcam (Cambridge, UK) were used: rabbit anti-rat GCalpha1 polyclonal antibody (catalogue-No. ab85445) at 0.25 µg/ml and rabbit anti-human GCbeta1 monoclonal antibody (clone EPR8822, catalogue-No. ab154841) at 1:10,000. Immunoreactive bands were detected using an enhanced chemiluminescence kit (Clarity Western ECL Substrate, Bio-Rad) and quantified using the ChemiDoc XRS+ system and Image Lab™ software (Bio-Rad). Equal loading was confirmed by stripping the membrane and reprobing it with an antibody against β-actin (1:5,000, MAB1501R, Merck Millipore).
| Gene/GenBank accession number | Direction | Sequence (5'-3')          |
|------------------------------|-----------|---------------------------|
| NOS1/ NM_052799.1             | forward   | GATCGGCGTCCGTGACTAC       |
|                              | reverse   | AGCAATGTTGATCTCCACCAGT    |
| NOS2/ NM_012611.3             | forward   | TGCTCCATAGTTTTTCAGAAGCAG  |
|                              | reverse   | ACTACTACCAGATCGAGCCCTG    |
| NOS3/ NM_021838.2             | forward   | CATGGAAAGGAAGTGCAAGCA     |
|                              | reverse   | AGCTGCTGTGCGTAGCTCT       |
| Ywhaz NM_013011               | forward   | CATCTGCAACGACGTACTGTCTCT  |
|                              | reverse   | CACAATTCCCTTTCTTGTATCACCA |
Figure S1. Sequence of interventions in acute experiments on renal function in anesthetized animals (experiments 1 and 2). Ach: acetylcholine

**Experiment 1**

- **Ach, dose response 1**
- Clearance 1
- Ach, dose response 2
- Clearance 2

**Time, min**

0 30 40 70 100 130 140 170

**Experiment 2**

- Blood sampling, L-NAME 10 mg/kg i.v.
- Clearance 1
- Clearance 2
- Blood sampling

**Time, min**

0 30 40 70
Figure S2. Renal function data from both kidneys obtained after L-NAME administration to the left kidney.

Glomerular filtration rate (GFR), natriuresis (UNaV) and diuresis measured simultaneously in the left kidney (L), which was infused with L-NAME via the renal artery at 250 µg/kg and in the right kidney (R) which did not receive L-NAME. White bars: Control animals. Shaded bars: Sunitinib-treated animals. (n = 10 per group) * p< 0.05
Figure S3. Renal nitric oxide synthase (NOS) mRNA abundances in control and four-day sunitinib-treated rats (n = 6-8 per group).

White bars: Control animals. Shaded bars: Sunitinib-treated animals.
Figure S4. Whole Western blots for renal cortical (A) and medullary (B) α₁ and β₁ subunits of soluble guanylate cyclase (sGC) as well as β-actin in control and four-day sunitinib-treated rats (n = 8 per group). C = control, S = sunitinib.
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