ONLINE SUPPLEMENT

SOD1 Limits Renal Microvascular Remodeling and Attenuates Arteriole and Blood Pressure Responses to Angiotensin II via Modulation of Nitric Oxide Bioavailability

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Supplemental Methods

Genotyping

From all SOD1-tg and SOD1-ko offspring a piece of the tail was collected for DNA preparation. Each sample tube contained Tris buffer (50mM TrisBase, 100mM ethylenediaminetetraacetic acid [EDTA], and 100mM NaCl, 20% sodium dodecyl sulfate, pH 7.5) with 10 mg/ml Proteinase K (Sigma-Aldrich Sweden AB) and was incubated at 55ºC overnight. Samples were then vortexed and centrifuged at 13,000 3 g for 10 min. To precipitate DNA, 300 µl of each supernatant was transferred to a new tube and mixed with 200 µl of 2-propanol (Kebo lab AB, Spånga, Sweden) and gently shaken. Next, the samples were centrifuged at 13,000 x g for 10 min. The supernatant was discarded and 1 ml of 70% ethanol was added to the pellets. Samples were then centrifuged at 13,000 x g for 5 min. Subsequently, the supernatant was removed and the pellets were dried and resuspended in RNase-free water. Finally the DNA concentration was estimated by measuring the absorbance of the samples.

Transgenic: Extracted DNA was amplified in a final volume of 10 µl reaction mix. Each reaction mix contained 5.80 µl of RNase-free water, 25mM $MgCl_2$, 10x PCR gold buffer, 2.5mM deoxy nucleotidyl triphosphate (dNTP) (Ampli Taq Gold with Gene Amp, Applied Biosystems, Branchburg, NJ), 20 µM of each mouse CuZnSOD and human CuZnSOD sense and antisense primers (TIB, Molbiol, Berlin, Germany) were used in the PCR reaction. Wild-type genotype is denoted if only the mouse endogenous CuZnSOD PCR product is amplified (product size of 324 bp). Heterozygous genotype is denoted if similar amounts of both the hCuZnSOD and mouse endogenous CuZnSOD PCR product are amplified (product size of 324 and 236 bp, respectively). Homozygous genotype is denoted if a higher amount of the inserted hCuZnSOD PCR product is amplified.

Knockout: Extracted DNA was amplified in a final volume of 10 µl reaction mix. Each reaction mix contained 15.3 µl of RNase-free water, 25mM MgCl2, 10x PCR gold buffer, 5mM dNTP (Ampli Taq Gold with Gene Amp), 20 µM of each HPRT (hypoxanthine guanine phosphoribosyl transferase), and CuZnSOD sense and antisense primers (TIB, Molbiol, Berlin, Germany). The HPRT primers amplify a DNA fragment, which includes the CuZnSOD promoter region and exon 2, whereas the primers for mouse endogenous CuZnSOD amplify a DNA fragment from the wt allele on exon 2. Wild-type genotype is denoted if only the mouse endogenous CuZnSOD PCR product is amplified (product size of 123 bp). Heterozygous genotype is denoted if PCR products positive for both HPRT and mouse CuZnSOD are amplified. Homozygous genotype is denoted if PCR products positive for only HPRT are amplified (product size of 240 bp).
Separate PCR cycling programs were used for tg and ko samples. The following parameters were used: Transgenic: (1) denaturation at 95ºC for 3 min, (2) amplification with a total of 35 cycles, each cycle with denaturation temperature at 95ºC for 30 s, annealing temperature at 60ºC for 30 s, elongation temperature at 72ºC for 2 min. Knock-out: (1) denaturation at 94ºC for 3 min, (2) amplification with a total of 35 cycles, each cycle with denaturation temperature at 94ºC for 30 s, annealing temperature at 61ºC for 1 min, elongation temperature at 72ºC for 2 min.

Telemetric Blood Pressure Measurements

Anaesthesia was induced by spontaneous inhalation of isoflurane (Forene®, Abbot Scandinavia AB, Kista, Sweden) and was continued throughout surgery by inhalation of 2.2% isoflurane in air. A midline incision was made between the lower mandible and sternum and the catheter of the telemetric blood pressure device (PA-C10 (DSI™, Transoma Medical, St Paul, MN, USA) was then inserted into the left carotid lumen and secured by 6/0 silk sutures (Silk®, Ethicon, Johnson & Johnson Intl, USA). The entry site was sealed by application of n-butyl-cyanoacrylate tissue adhesive (VetbondTM, 3M Animal Care Products, St Paul, MN, USA) and the body of the transmitter was placed subcutaneously in the right flank. Finally the skin incision was closed using 5/0 polyamide sutures (Ethilon® , Ethicon, Johnson & Johnson Intl). The animals were placed in new cages and were allowed to wake up under a heating lamp. All animals were allowed to recover for at least 10 days before the telemetric measurements were commenced. The implanted receiver was switched on, and the mouse cage was placed on a receiver plate which transferred the signals to a computer, where calibrated blood pressure and heart rate values were sampled. Data were collected for 5 seconds every second minute throughout the measurements.

Renal excretion of nitrate/nitrite

To ensure a low overall nitrate intake we first measured nitrate content in several standard rodent diets and found that it varied considerably (0.14-1.5 mM). The chow containing 0.14 mM nitrate was given to all animals. Mice were placed in metabolism cages for 24-hours, with food and water given ad libitum. Water consumption and urine production were measured gravimetrically. Samples of fresh urine were stored at -70ºC until analysis. Nitrate and nitrite in urine (diluted 50 times with carrier buffer) were measured with a dedicated high performance liquid chromatography (HPLC) system (ENO-20; EiCom, Kyoto, Japan).[1] The method is based on the separation of nitrate by reverse-phase/ion exchange chromatography, followed by on-line reduction of nitrate to nitrite with cadmium and reduced copper. Reduced nitrite was then derivatised with Griess reagent and the level of diazo compounds measured at 540 nm. The retention time was 7.0 minutes for nitrate and 4.5 minutes for nitrite.

Dissection and perfusion of arterioles

The outer cortical afferent arterioles were dissected at 4°C in Dulbecco's modified Eagle's medium (DMEM, 0.1% albumin). Arterioles with their glomeruli were perfused in a thermo-regulated chamber (37°C) by a perfusion system, which allowed adjustment of outer holding and inner perfusion pipettes (Vestavia Scientific, Vestavia Hills, AL, USA). The chamber and the perfusion system were fixed to the stage of an inverted microscope (Nikon, Badhoevedorp, Netherlands). A 5-μm diameter perfusion pipette was connected to a reservoir containing the perfusion solution to provide a pressure of 100 mmHg in the pressure head, which produced a flow of about 50 nl/min. The criteria for the use of an arteriole were: a satisfactory, remaining basal tone and an intact myogenic response. Both criteria were tested by increasing perfusion pressure rapidly and assessing the change in the luminal diameter, which produced a constriction. A further criterion was a fast and complete constriction in response to KCl (100 mmol/l) solution. Results from the afferent arteriole measurements are shown in Figure S1-S5).

Superoxide measurements in afferent arterioles
Superoxide generation was assessed by fluorescence microscopy of isolated and perfused afferent arterioles with dihydroethidium (DHE) or tempo-9-AC, as previously described.[2] DHE is freely permeable to cells and oxidized by O_2^- to the highly fluorescent compound ethidium, which is trapped intracellularly and intercalated into DNA. The conversion of DHE to ethidium was quantified by a dual wavelength determination using an excitation wavelength of 380 nm and an emission wavelength of 460 nm for DHE and an excitation wavelength of 480 nm and an emission wavelength of 605 nm for ethidium. Single-agent signal capture was achieved by cycling at 3-s intervals between a 460- and 605-nm filter. Changes in O_2^- were expressed as the ratio of ethidium:DHE fluorescence. The system used an Olympus IX70 fluorescence microscope equipped with dual photomultipliers (PMT, Photon Technology Int., Lawrenceville, NJ). Excitation was provided by a 75-W xenon arc lamp using a 380/460 nm wavelength combination isolated with a computer-controlled monochromator. Ethidium and DHE emit blue and red light, respectively, that were directed to a dual PMT assembly by a beam splitter that directed light to the two separate PMT using a 400-nm dichroic mirror and barrier filters centred at 460 and 605 nm, respectively. The ratio of ethidium:DHE was monitored in real time and recorded by software (Felix32; Photon Technology Int.). This ethidium fluorescence method has also been shown to detect a product that differs from ethidium by the presence of an additional oxygen atom in its molecular structure.[3] Therefore additional studies were undertaken using tempo-9-AC fluorescence to detect O_2^-.[4, 5] There was good agreement between the two methods to detect Ang II induced O_2^- generation in afferent arterioles.

Analysis of mRNA expression
Infusion of cold PBS (Phosphate Buffered Saline) was started once the vena cava was cut to remove the blood. The heart and kidneys were explanted, dried and weighed. The renal cortex was separated and homogenised in lysis buffer (1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 80mM Tris, pH 7.5) containing enzyme inhibitors (Complete Mini; 1 tablet/1.5 ml; Roche Diagnostics, Mannheim, Germany). RNA was isolated with RNA-Bee-reagent (Biozol, Eching, Germany) and reverse transcribed with random hexamers (High Capacity cDNA RT-Kit, Applied Biosystems, Foster City, CA, USA, #4374966), according to the manufacturer's protocols. Quantitative PCR analysis was performed with a StepOnePlus device (Applied Biosystems, Foster City, CA, USA). SYBR Green was used for the fluorescent detection of DNA generated during PCR. The PCR reaction was performed in a total volume of 12.5 µl with 0.4 pmol/µl of each primer (Table S1), and ImmoMix (Bioline, Luckenwalde, Germany): 2µl cDNA corresponding to 20 ng RNA was used as a template. Experiments were performed in triplicate with similar results. The expression levels of mRNA were normalised to β-actin by the ΔCt-method. Parallelism of amplification curves of the test and control was confirmed. Results from the mRNA analysis are shown in Table S2 and Figure S6.

Drugs and Reagents

DMEM/F12 with 10mmol/l HEPES (Invitrogen AB, Lidingö, Sweden) was used for dissection, bath, and perfusion. The pH was adjusted to 7.4 after addition of bovine serum albumin (BSA: SERVA Electrophoresis Heidelberg, Germany). The concentration of BSA was 0.1% in dissection and bath solutions, and 1% in the perfusion solution. The K^+ solution had the composition in mmol/l, NaCl 20, KCl 95, $NaHCO_3$ 25, K_2HPO_4 2.5, $CaCl_2$ 1.3, $MgSO_4$ 1.2, glucose 5.5, and was equilibrated with 5% CO_2 in air.

The drugs used were: Angiotensin II, Nω-Nitro-L-arginine methyl ester hydrochloride (L NAME), and 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (tempol) from Sigma-Aldrich. All drugs were applied to the bath solution in the arteriolar contraction experiments.

Supplemental References

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2. Wang D, Jose P, Wilcox CS. beta(1) Receptors protect the renal afferent arteriole of angiotensin-infused rabbits from norepinephrine-induced oxidative stress. *J Am Soc Nephrol.* 2006;17:3347-3354.

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4. Fellner SK, Arendshorst W. Endothelin-A and -B receptors, superoxide, and Ca2+ signaling in afferent arterioles. *Am J Physiol Renal Physiol.* 2007;292:F175-184.

5. Fellner SK, Arendshorst WJ. Angiotensin II, reactive oxygen species, and Ca2+ signaling in afferent arterioles. *Am J Physiol Renal Physiol.* 2005;289:F1012-1019.

Table S1. Primers used for PCR analysis

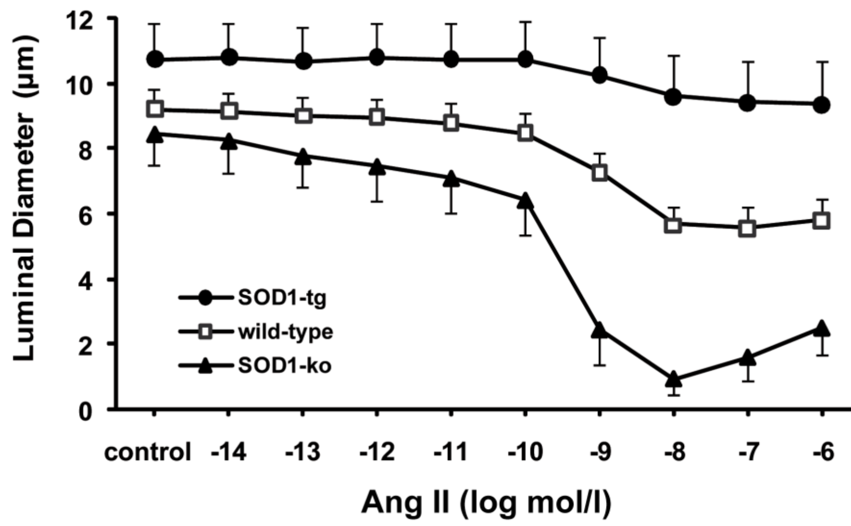
Number	Primer Name	Primer Sequence
NM_011435	SOD1-fw	5'- TCC CTT CGA GCA GAA GGC AAG C -3'
	SOD1-rev	5'- ACA TGC CTC TCT TCA TCC GCC G -3'
NM_013671	SOD2-fw	5'- ACG CGG CCT ACG TGA ACA ATC TC -3'
	SOD2-rev	5'- CAA CTC TCC TTT GGG TTC TCC ACC A -3'
NM_011434	SOD3-fw	5'- CTC TAG CTG GGT GCT GGC CTG AAC T -3'
	SOD3-rev	5'- AAC CGG GTC AAG CCT GTC TGC TAG -3'
NM_177322	AT1a-fw	5'- GAT TGG TAT AAA ATG GCT GG -3'
	AT1a-rev	5'- TCT GGG TTG AGT TGG TCT CA -3'
NM_175086	AT1b-fw	5'- GGG AGT AGG GAT CAT GAC AA -3'
	AT1b-rev	5'- CAC TGT AGA TGG GGA GCA GCC AA -3'
NM_007429	AT2-fw	5'- GGA CTC ATT GGT GCC AGT TG -3'
	AT2-rev	5'- GCT TAC TTC AGC CTG CAT TT -3'
NM_008713	eNOS-fw	5'- GTT TGT CTG CGG CGA TGT C -3'
	eNOS-rev	5'- CAT GCC GCC CTC TGT TG -3'
NM_070927	iNOS-fw	5'- GGC AGC CTG TGA GAC CTT TG -3'
	iNOS-rev	5'- CAT TGG AAG TGA AGC GTT TCG -3'
NM_008712	nNOS-fw	5'- TCG GCT GTG CTT TGA TGG A -3'
	nNOS-rev	5'- TTG AAT CGG ACC TTG TAG CTC TTC -3'
NM_172203	Nox1-fw	5'- TCA CGA GTG GGA TGA CCA TAA GGG G -3'
	Nox1-rev	5'- CTC GCT TCC TCA TCT GCA ATT CCA A -3'
NM_007807	Nox2-fw	5'- GCA CCT GCA GCC TGC CTG AAT T -3'
	Nox2-rev	5'- TTG TGT GGA TGG CGG TGT GCA -3'
NM_015760	Nox4-fw	5'- GGC TGG CCA ACG AAG GGG TTA A -3'
	Nox4-rev	5'- GAG GCT GCA GTT GAG GTT CAG GAC A -3'
NM_007806	p22Phox-fw	5'- CTG GCG TCT GGC CTG ATT CTC ATC -3'
	p22Phox-rev	5'- CCG AAA AGC TTC ACC ACA GAG GTC A -3'
NM_010876	p47Phox-fw	5'- CAG CCA TGG GGG ACA CCT TCA TT -3'
	p47Phox-rev	5'- GCC TCA ATG GGG AAC ATC TCC TTC A -3'
NM_010877	p67Phox-fw	5'- AAG ACC TTA AAG AGG CCT TGA CGC A -3'
	p67Phox-rev	5'- TCG GAC TTC ATG TTG GTT GCC AA -3'

Table S2. Fold changes in mRNA expression compared with wild-type mice.

Gene	Wild-type	SOD1-tg	SOD1-ko
SOD1	1.00 ± 0.43	1.44 ± 0.42	0.00 ± 0.00
SOD2	1.00 ± 0.47	1.43 ± 0.44	1.51 ± 0.41
SOD3	1.00 ± 0.26	1.03 ± 0.18	1.49 ± 0.24
AT_{1A}	1.00 ± 0.20	0.80 ± 0.28	0.79 ± 0.26
AT_{1B}	1.00 ± 0.61	2.24 ± 1.07	0.92 ± 0.49
AT_2	1.00 ± 0.48	1.96 ± 0.78	0.60 ± 0.37
eNOS	1.00 ± 0.10	1.65 ± 0.22	1.98 ± 0.52
iNOS	1.00 ± 0.73	3.44 ± 1.76	4.20 ± 1.80
nNOS	1.00 ± 0.29	0.88 ± 0.37	1.98 ± 0.83
NOX1	1.00 ± 0.65	3.58 ± 1.40	4.05 ± 2.17
NOX2	1.00 ± 0.35	1.05 ± 0.12	1.64 ± 0.49
NOX4	1.00 ± 0.31	2.12 ± 0.22	1.10 ± 0.33
p22phox	1.00 ± 0.21	0.71 ± 0.12	1.29 ± 0.35
p47phox	1.00 ± 0.24	1.45 ± 0.15	1.68 ± 0.53
p67phox	1.00 ± 0.37	0.98 ± 0.37	0.79 ± 0.25

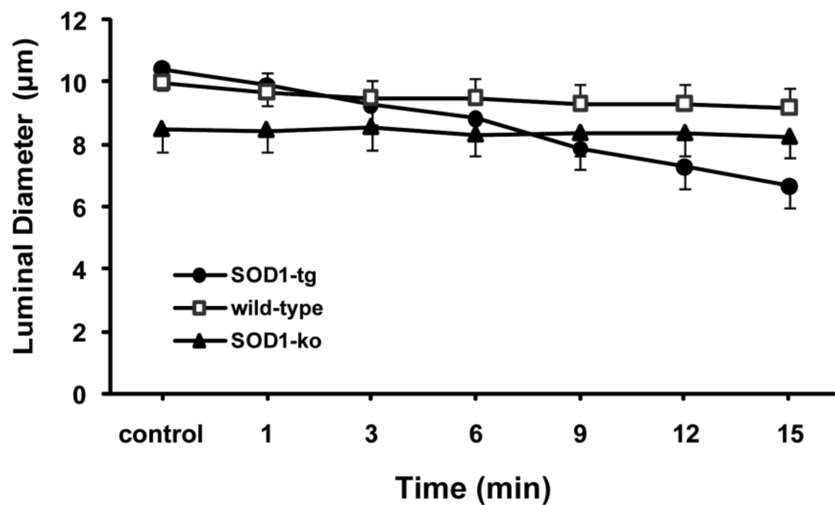
Superoxide dismutase isoforms (SOD1, SOD2, SOD3); Angiotensin I receptors ($AT_{1A,}$ $AT_{1B,}$ AT_2); nitric oxide synthase isoforms (eNOS, iNOS, nNOS); and NADPH-oxidase isoforms (NOX1, NOX2, NOX4) and subunits (p22Phox, p47Phox, p67Phox) in the renal cortex of non-treated wild-type, SOD1-transgenic (SOD1-tg), and SOD1-knockout (SOD1-ko) mice. Fold changes are expressed as mean ± standard error

Figure S1.



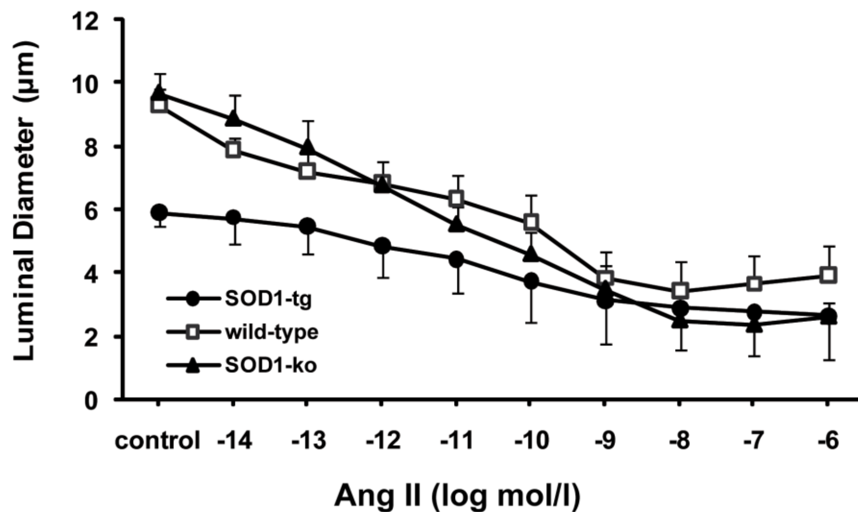
Ang II concentration response curves in isolated and perfused afferent arterioles of SOD1-transgenic (SOD1-tg; n=7), wild-type (n=11), and SOD1-knockout (SOD1-ko, n=7) mice. Control period represents the arteriolar diameter before Ang II application.

Figure S2.



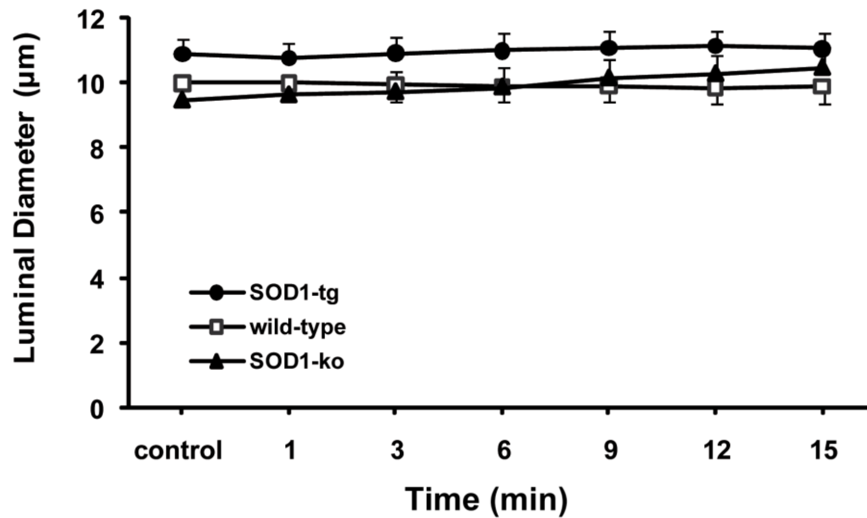
Effect of L-NAME (10^{-4} mol/l) on diameters of isolated and perfused afferent arteriolar from SOD1-transgenic (SOD1-tg; n=7), wild-type (n=8), and SOD1-knockout (SOD1-ko; n=8) mice.

Figure S3.



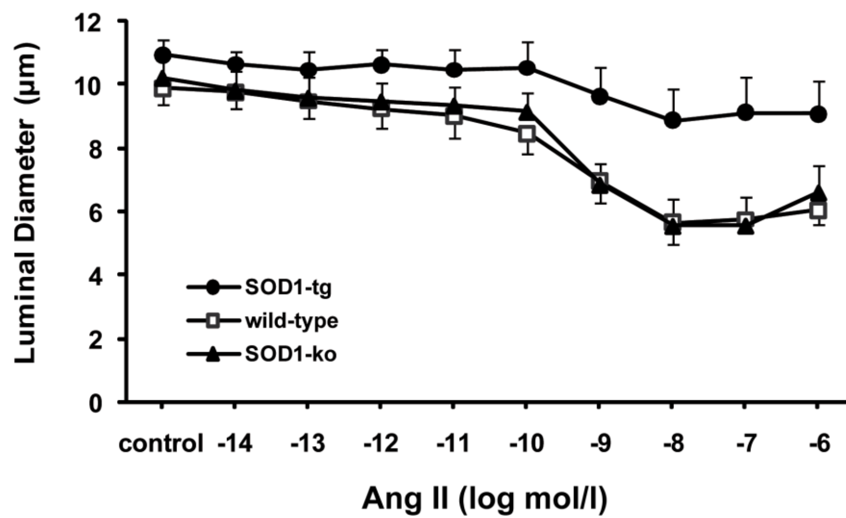
Effect of L-NAME (10^{-4} mol/l) on Ang II concentration response in isolated and perfused afferent arterioles of SOD1-transgenic (SOD1-tg; n=10), wild-type (n=8), and SOD1-knockout (SOD1-ko; n=7) mice. Control period represents the arteriolar diameter after L-NAME pre-treatment for 15 min.

Figure S4.



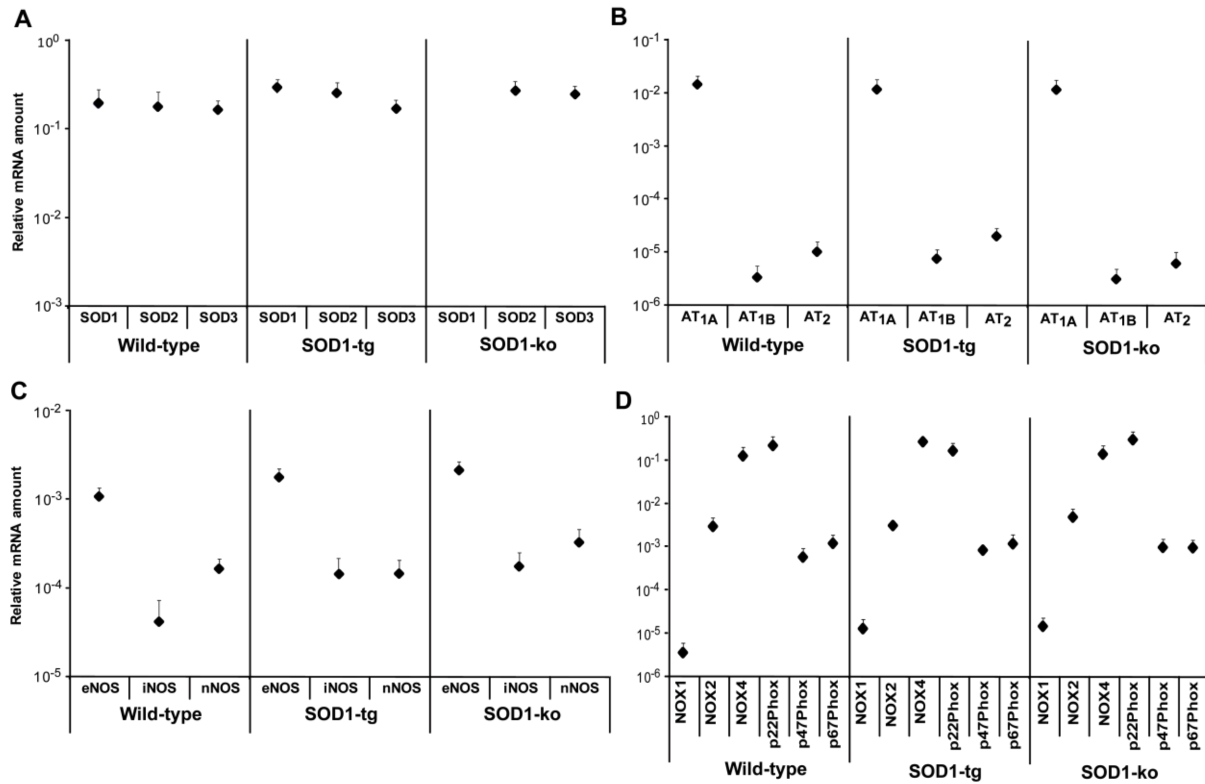
Effect of tempol (10^{-4} mol/l) on diameters of isolated and perfused afferent arteriolar from SOD1-transgenic (SOD1-tg; n=6), wild-type (n=7), and SOD1-knockout (SOD1-ko; n=7) mice.

Figure S5.



Effect of tempol (10^{-4} mol/l) on Ang II concentration response in isolated and perfused afferent arterioles of SOD1-transgenic (SOD1-tg; n=6), wild-type (n=7), and SOD1-knockout (SOD1-ko; n=7) mice. Control period represents the arteriolar diameter after tempol pre-treatment for 15 min.

Figure S6.



Relative mRNA expression of (A) superoxide dismutase isoforms (SOD1, SOD2, SOD3); (B) Angiotensin I receptors (AT_{1A}, AT_{1B}, AT_2); (C) nitric oxide synthase isoforms (eNOS, iNOS, nNOS); and, (D) NADPH-oxidase isoforms (NOX1, NOX2, NOX4) and subunits (p22Phox, p47Phox, p67Phox) in the renal cortex of non-treated wild-type, SOD1-transgenic (SOD1-tg), and SOD1-knockout (SOD1-ko) mice.