DATA SUPPLEMENT

Knockout of Macula Densa NOS1 Increases Blood Pressure in db/db Mice

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METHODS

Measurement of GFR in Conscious Mice

GFR was measured via determination of plasma FITC-sinistrin clearance kinetics in conscious mice as we previously described.[1-3] Mice were lightly anesthetized with isoflurane and injected with a single bolus of FITC-sinistrin solution (5.6 mg/100 g body weight) via the retro-orbital venous sinus. Blood samples (~10 µl/each) were collected into heparinized capillary tubes from the tail vein at 3, 7, 10, 15, 35, 55, 75 and 90 minutes after injection. The blood samples were centrifuged at 8000 rpm for 5 minutes at 4˚C to separate plasma (~5 µl/each). The fluorescence intensity of FITC-sinistrin in the plasma sample was measured using a plate reader (Cytation3, BioTek, VT) with 485-nm excitation and 538-nm emission. The GFR value was calculated with GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA).

Measurement of TGF *in vivo* with Micropuncture

In vivo TGF response was measured in anesthetized mice using micropuncture techniques as we previously described.[1-4] Mice were anesthetized with inactin (70 mg/kg, i.p.) plus ketamine (50 mg/kg, i.m.), and maintained throughout the experiment on a homeothermic table set at 37 ˚C. A tracheostoma was placed to facilitate respiration. Blood pressure was monitored via a pressure transducer (ADInstruments, Sydney, Australia) placed in the right femoral artery. The left femoral vein was catheterized to infuse saline solution with 2% bovine serum albumin (BSA) at a rate of 1 ml/h per 100 g body weight throughout the experiment. The left kidney was exposed via an abdominal incision, and secured in a kidney holder. A superficial proximal tubule with multiple visible loops was visualized (Olympus SZX16; Tokyo, Japan), and then obstructed by inserting a grease block in the loop. The stop-flow pressure (P_{sf}) in the proximal tubule upstream of the grease block was measured by the servo-null method with a pressure micropipette (Model 900A; World Precision Instruments, Sarasota, FL). The segment of the proximal tubule distal to the grease block was perfused with artificial tubular fluid (ATF, containing 4 mM $NaHCO_3$, 5 mM KCl, 2 mM $CaCl_2$, 7 mM urea, 2 mM $MgCl_2$, 128 mM NaCl, and 1% fast green; pH 7.4) via a perfusion micropipette. The P_{sf} was continuously recorded while the tubular perfusion rate was switched from 0 to 40 nl/min. The change in the P_{sf} (ΔP_{sf}) was used as the index of TGF response *in vivo*.

Measurement of TGF *in vitro* in Isolated/Perfused JGA

In vitro TGF response was measured in isolated and perfused mouse JGAs as we previously described.[1-4] Briefly, a single afferent arteriole (Af-Art) with its intact glomerulus and adherent tubular segments [consisting of the thick ascending limb (TAL), macula densa, and early distal tubule] were microdissected from fresh mouse kidney slices within 30 minutes in ice-cold DMEM containing 5% BSA under a stereomicroscope (SMZ1500; Nikon, Yuko, Japan). The isolated JGA was then transferred to a temperature-regulated chamber mounted on an inverted microscope (Axiovert 100TV, ZEISS, NY). The bath solution had a total volume of 1.5 ml that was continuously exchanged at a rate of 1 ml/min and maintained at a temperature of 37 °C throughout the experiment. The Af-Art was cannulated with a set of glass pipettes and perfused with DMEM at 60 mmHg. The TAL was cannulated with another set of glass pipettes and perfused with an artificial macula densa perfusate fluid containing 10 mM HEPES, 1.0 mM $Ca(HCO_3)_2$, 0.5 mM K_2HPO_4, 4.0 mM $KHCO_3$, 1.2 mM $MgSO_4$, 5.5 mM glucose, 0.5 mM Na acetate, 0.5 mM Na lactate, 0.5 mM l-arginine, and either 10 or 80 mM NaCl (pH: 7.4). Once perfused, the JGA was subjected to a 30-minute equilibration period. The luminal diameter of Af-Art was then continuously recorded as the macula densa perfusate was switched from 10 mM NaCl to 80 mM NaCl. The change in the luminal diameter of Af-Art was used as the index of TGF response *in vitro*.

Measurement of NO generation in the Macula Densa of Isolated/Perfused JGA

NO generation in the macula densa of isolated/perfused mouse JGA was measured using the fluorescent NO probe 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-2 DA) as we previously described.[1-4] Isolation and perfusion of the JGA was performed as the description in the previous section. After the equilibration period, tubular segment was loaded via luminal administration of DAF-2 DA (10 μM plus 0.1% pluronic acid) for 30 minutes, followed by a 15-minute washout period using macula densa perfusate fluid. The DAF-2 was excited at 490 nm and the emitted fluorescence was recorded at wavelengths of 510 to 550 nm. The rate of increase in the fluorescent intensity of DAF-2 was used as the index of NO generation. The imaging system was composed of a microscope (Eclipse Ti; Nikon), a digital charge-coupled device camera (CoolSnap; Photometrics, Tucson, AZ), a xenon light (LB-LS/30; Shutter Instruments), and an optical filter changer (Lambda 10-3; Shutter Instruments). Images were displayed and analyzed with NIS-Elements imaging software (Nikon).

Measurement of NOS1 Expression and Phosphorylation by Western Blot

The expression and phosphorylation levels of NOS1 were measured by western blot as we previously described.[1-4] Briefly, protein extracts from renal cortex were separated on 7.5% SDS-PAGE gels (50 μg per lane) and then transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk for 1 hour at room temperature and then incubated with an antibody for NOS1 (C–terminal) (610309; mouse polyclonal IgG; 1:3000; BD Biosciences, San Jose, CA) or an antibody for NOS1 phosphorylated at Ser1417 (P-NOS1) (ab5583; rabbit polyclonal IgG; 1:500; Abcam) overnight at 4°C. Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (P044701-2; goat anti-mouse IgG/HRP; 1:300,000 or P044801-2; goat anti-rabbit IgG/HRP; 1:300,000; Agilent Technologies, Santa Clara, CA) for 1 hour at room temperature. The immunoreactive bands were detected using ChemiDoc System (Bio-Rad, Hercules, CA) and quantified using ImageLab software (Bio-Rad, Hercules, CA). Normalization was performed by stripping the membranes with the Restore Western Blot Stripping Buffer (Fisher Scientific, Waltham, MA) for 15 minutes at room temperature and incubating them with a β-actin antibody (A5441; mouse monoclonal IgG; 1:5000; Sigma, St. Louis, MO).

Identification of Tissue-Specific NOS1 Expression by Double-Immunofluorescence Staining

Double-immunofluorescence staining of NOS1 and NKCC2 in kidney sections was performed as we previously described.[1, 2, 5] Briefly, 2 μm slices were incubated overnight (at 4°C) with both a NOS1 antibody (BML-SA227-0100; rabbit polyclonal IgG, 1:200; Enzo Biochem, Farmingdale, NY) and a NKCC2 antibody (sc-293222; mouse monoclonal IgG, 1:200; Santa Cruz Biotechnology, Dallas, TX). This was followed by a 1-hour incubation (at room temperature) with fluorescent secondary antibodies (ab150080; Alexa Fluor 594 goat anti-rabbit IgG, 1:1000; and ab150113; Alexa Fluor 488 goat anti-mouse IgG, 1:1000; Abcam, Cambridge, United Kingdom). Negative controls included sections incubated without primary and/or secondary antibodies. All slices were set in an antifade mounting medium with DAPI (H-1500; Vector Laboratories, Burlingame, CA). All images were captured with Nikon Eclipse E600FN confocal microscope equipped with Photometrics Cascade 512F digital camera.

Measurement of Mean Arterial Pressure (MAP) in Conscious Mice

MAP was measured with radio telemetry system (PA-C10; Data Sciences International, MN, USA) in conscious mice.[6] Mice were anesthetized with inhaled isoflurane via vaporizer (Vaporizer Sales & Service, Rockmart, GA). Under aseptic conditions, a small incision was made in the middle of the neck to expose the left carotid artery. The pressure catheter of transmitter was

inserted into the left carotid artery and further advanced down to the aortic arch. Then, the body of transmitter was placed subcutaneously in the right ventral flank. The mice were allowed 7 days of recovery before collecting data. MAP was recorded for 10 seconds every 1 minute for 4 hours each day from 1 to 5 post meridian.

Measurement of Biochemical Parameters

Urine samples were collected by picking up the mice to elicit reflex urination and holding them over a petri dish. Blood samples were collected through the retroorbital venous sinus immediately after urine collection.[7] Urinary creatinine concentration (UCr) and plasma creatinine concentration (PCr) were measured by HPLC at the O'Brien Center Core of the University of Alabama at Birmingham.[8, 9] Urinary Na^+ concentration (UNa) and plasma Na^+ concentration (PNa) were measured by a photoelectric flame photometer (BWB Technologies, Hambridge, United Kingdom). The FENa was calculated with the following formula: FENa (%) = (UNa X PCr) / (PNa X UCr) X 100.

Measurement of Renal Superoxide (O_2^-) Production

The renal O_2^- production was detected with dihydroethidium (DHE) staining. Briefly, fresh frozen kidney sections were incubated with 1 mM DHE for 15 minutes at 37 °C and then washed for 5 minutes with PBS. All sections were set in an antifade mounting medium (H-1000-10; Vector Laboratories, Burlingame, CA), and all images were captured with Nikon Eclipse E600FN confocal microscope equipped with Photometrics Cascade 512F digital camera.

Measurement of Renal Hydrogen Peroxide (H_2O_2) Production

The H_2O_2 level in kidney homogenates was measured by fluorometric assay with a highly sensitive kit (ab102500; Abcam) according to the manufacturer's protocol. The fluorescence was detected at Ex/Em = 535/587 nm using a microplate reader (Cytation3, BioTek, VT).

Measurement of Renin Protein Expression by Western Blot

Total proteins were extracted from renal cortical tissue, separated on 7.5% SDS-PAGE gels (50 μg per lane) and then transferred to nitrocellulose membranes. The membranes were incubated with a renin antibody (sc-133145; mouse monoclonal IgG; 1:500; Santa Cruz, Dallas, TX) overnight at 4°C followed by a horseradish peroxidase-conjugated secondary antibody (P044701-2; goat anti-mouse IgG/HRP; 1:300,000) for 1 hour at room temperature. The immunoreactive bands were detected using ChemiDoc System (Bio-Rad, Hercules, CA) and quantified using ImageLab software (Bio-Rad, Hercules, CA). Normalization was performed by stripping the membranes with the Restore Western Blot Stripping Buffer (Fisher Scientific, Waltham, MA) and then staining for β-actin (A5441; mouse monoclonal IgG; 1:5000; Sigma, St. Louis, MO).

Measurement of Renin mRNA Expression by Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNAs were extracted from renal cortical tissue with the RNeasy Mini Kit (74104; Qiagen, Hilden, Germany) and then reverse-transcribed to cDNAs with the RevertAid First Strand cDNA Synthesis Kit (FERK1622; Thermo Fisher Scientific, Waltham, MA) as we previously described.[1, 6] Quantitative PCR was performed using the Maxima SYBR Green Master Mix (FERK0221, Thermo Fisher Scientific) and Real-Time Detection System (Chromo4, Bio-Rad, CA, USA) according to the manufacturer's protocol. The condition of the reaction was set as following: 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds. The data were analyzed with the comparative Ct method ($2^{-\Delta\Delta Ct}$). The primer sequences are listed in Supplemental Table S1.[10, 11]

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FIGURES

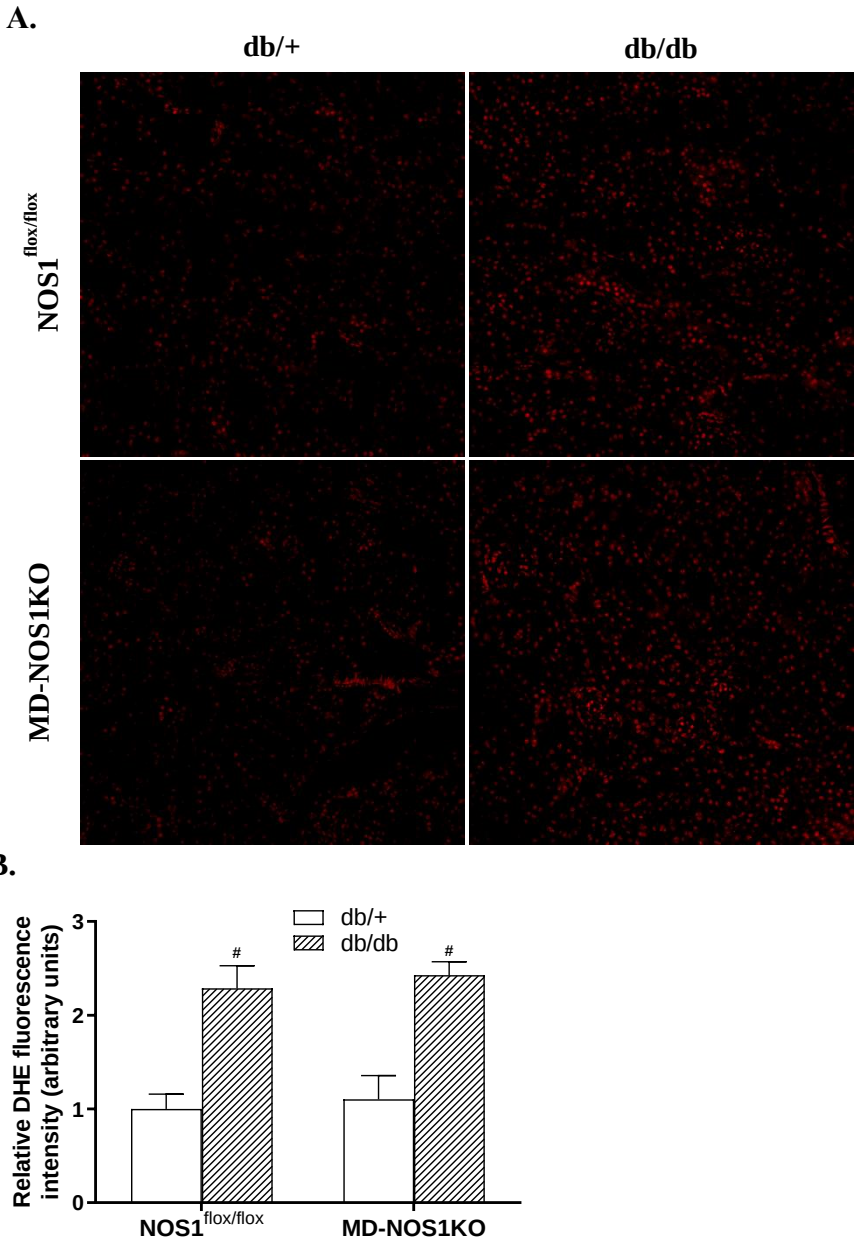


Figure S1. Effect of macula densa NOS1 deletion on renal O_2^- production in db/+ and db/db mice. (A) Representative images of the DHE staining in kidney sections of db/+ NOS1$^{flox/flox}$, db/db NOS1$^{flox/flox}$, db/+ MD-NOS1KO and db/db MD-NOS1KO mice. **(B)** Quantification of the renal DHE fluorescence intensity (O_2^- level) in db/+ NOS1$^{flox/flox}$, db/db NOS1$^{flox/flox}$, db/+ MD-NOS1KO and db/db MD-NOS1KO mice. n=10 sections/per mice, 8 mice/per group; $^{\#}P < 0.01$ vs db/+.

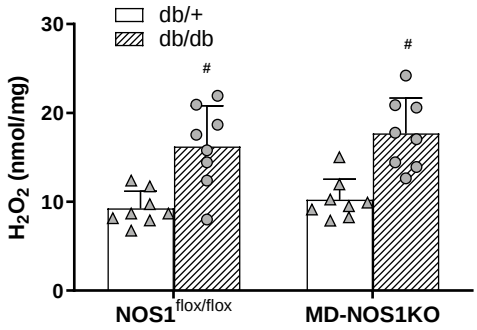
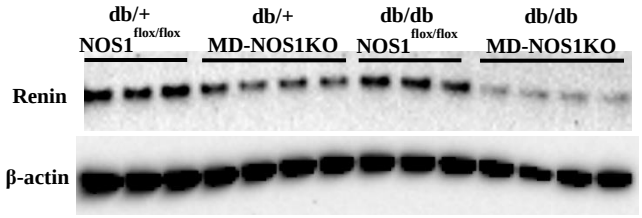


Figure S2. Effect of macula densa NOS1 deletion on renal H_2O_2 generation in db/+ and db/db mice. The H_2O_2 levels in kidney homogenates of db/+ NOS1$^{flox/flox}$, db/db NOS1$^{flox/flox}$, db/+ MD-NOS1KO and db/db MD-NOS1KO mice. n=8; $^{\#}P < 0.01$ vs db/+.

A.



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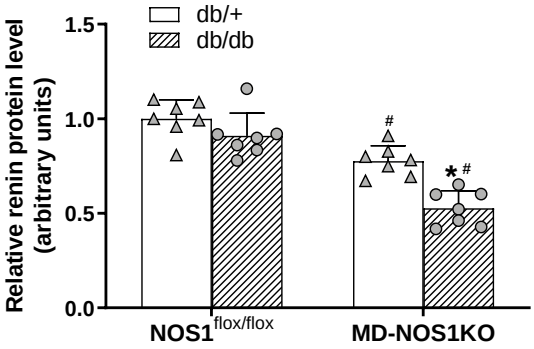

C.

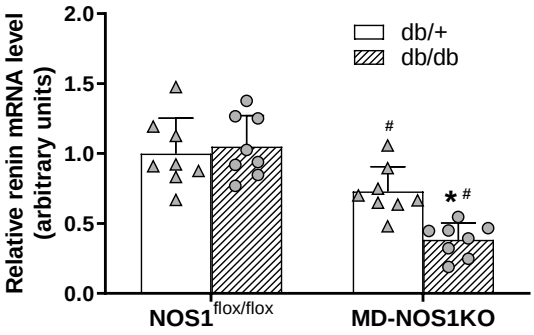

Figure S3. Effect of macula densa NOS1 deletion on renal renin expression in db/+ and db/db mice. (A) The immunoblots of renal renin and loading control of β-actin in db/+ $NOS1^{flox/flox}$, db/db $NOS1^{flox/flox}$, db/+ MD-NOS1KO and db/db MD-NOS1KO mice. **(B)** The renal cortical renin protein levels in db/+ $NOS1^{flox/flox}$, db/db $NOS1^{flox/flox}$, db/+ MD-NOS1KO and db/db MD-NOS1KO mice. n=7; *P<0.01 vs db/+, $^{\#}$P<0.01 vs $NOS1^{flox/flox}$. (**C**) The renal cortical renin mRNA levels in db/+ $NOS1^{flox/flox}$, db/db $NOS1^{flox/flox}$, db/+ MD-NOS1KO and db/db MD-NOS1KO mice. n=8; *P<0.05 vs db/+, $^{\#}$P<0.01 vs $NOS1^{flox/flox}$.

Table S1. Kidney weight.

Groups	db/+	db/db	db/+ NOS1$^{flox/flox}$	db/db NOS1$^{flox/flox}$	db/+ MD-NOS1KO	db/db MD-NOS1KO
Kidney Weight (g)	0.268±0.009	**0.404**±0.012*	**0.280**±0.010	**0.396**±0.011*	**0.288**±0.009	**0.344**±0.014*$^{\#}$

*p<0.01 vs. db/+; $^{\#}$p<0.01 vs. NOS1$^{flox/flox}$;
Two-way ANOVA for db/+ NOS1$^{flox/flox}$, db/db NOS1$^{flox/flox}$, db/+ MD-NOS1KO, and db/db MD-NOS1KO groups. P value (interaction)<0.01, P value (NOS1)<0.01, P value (diabetes)<0.01

Table S2. Primer sequences.

Gene	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
β-actin[10]	GTCCCTCACCCTCCCAAAAG	GCTGCCTCAACACCTCAACCC
Ren1[11]	ACAGTATCCCAACAGGAGAGACAAG	GCACCCAGGACCCAGACA