Aldosterone Blunts Tubuloglomerular Feedback by Activating Macula Densa Mineralocorticoid Receptors

Yiling Fu, John E. Hall, Deyin Lu, Lin Lin, R. Davis Manning, Jr, Liang Cheng, Celso E. Gomez-Sanchez, Luis A. Juncos, Ruisheng Liu

Abstract—Chronic aldosterone administration increases glomerular filtration rate, whereas inhibition of mineralocorticoid receptors (MRs) markedly attenuates glomerular hyperfiltration and hypertension associated with primary aldosteronism or obesity. However, the mechanisms by which aldosterone alters glomerular filtration rate regulation are poorly understood. In the present study, we hypothesized that aldosterone suppresses tubuloglomerular feedback (TGF) via activation of macula densa MR. First, we observed the expression of MR in macula densa cells isolated by laser capture microdissection and by immunofluorescence in rat kidneys. Second, to investigate the effects of aldosterone on TGF in vitro, we microdissected the juxtaglomerular apparatus from rabbit kidneys and perfused the afferent arteriole and distal tubule simultaneously. Under control conditions, TGF was 2.8 ± 0.2 μm. In the presence of aldosterone (10⁻⁸ mol/L), TGF was reduced by 50%. The effect of aldosterone to attenuate TGF was blocked by the MR antagonist eplerenone (10⁻⁵ mol/L). Third, to investigate the effect of aldosterone on TGF in vivo, we performed micropuncture, and TGF was determined by maximal changes in stop-flow pressure Psf when tubular perfusion rate was increased from 0 to 40 nl/min. Aldosterone (10⁻⁷ mol/L) decreased ΔPsf from 10.1 ± 1.4 to 7.7 ± 1.2 mm Hg. In the presence of L-NG-monomethyl arginine citrate (10⁻³ mol/L), this effect was blocked. We conclude that MRs are expressed in macula densa cells and can be activated by aldosterone, which increases nitric oxide production in the macula densa and blunts the TGF response.

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Key Words: tubuloglomerular feedback ■ aldosterone ■ mineralocorticoid receptor ■ nitric oxide
of NaCl reabsorption in distal nephron segments by MR antagonism would require decreased distal delivery of NaCl, possibly through reductions in GFR, to achieve salt balance if intake was unaltered. TGF resetting could be an important mechanism for mediating reduced GFR and distal NaCl delivery during MR antagonism.

Despite the potential adaptive value of TGF resetting and glomerular hyperfiltration in offsetting the renal sodium retaining actions of aldosterone and MR activation, this effect has been proposed to contribute to renal injury in patients with primary aldosteronism. Although MR activation by aldosterone is recognized to be an important cause of glomerular hyperfiltration, the mechanisms involved are poorly understood. In preliminary studies we found significant MR expression on macula densa (MD) cells, suggesting a potential role in TGF. To our knowledge, the direct effects of aldosterone on TGF have not been reported previously. Therefore, in the present study we tested the hypothesis that aldosterone suppresses TGF through MR activation in MD cells. Multiple approaches were used to test this hypothesis, including investigation of the effects of aldosterone on TGF using microdissected and perfused juxtaglomerular apparatus (JGA) in vitro and micropuncture studies in vivo. Our studies reveal that aldosterone reduces TGF by stimulation of nitric oxide (NO) synthesis and that this effect is mediated through activation of MR.

Methods

All of the procedures and experiments were approved by the institutional animal care and use committee at the University of Mississippi Medical Center. All of the chemicals were purchased from Sigma (St Louis, MO) except as indicated.

Microperfusion: Isolation and Microperfusion of the Rabbit Afferent Arteriole and Attached MD
We used methods similar to those described previously (please see the online-only Data Supplement). After the 30-minute equilibration period, the MD perfusate was switched from 10 to 80 mmol/L of NaCl at a rate of 40 nL/min, and luminal diameter of the afferent arteriole (Af-Art) perfused at 60 mm Hg was measured for 5 minutes. We used the average change in diameter of the Af-Art as our control TGF response. Then the MD perfusate was switched back to 10 mmol/L of NaCl. To study the effect of aldosterone on regulation of the TGF response in vitro, aldosterone (10^{-8} mol/L) was added to the tubular perfusate for 15 minutes, and a second TGF response was measured. To determine whether the aldosterone-induced TGF alteration could be inhibited by MR blockade, a selective MR antagonist, eplerenone, 10^{-3} mol/L, was added to the tubular perfusate 30 minutes before aldosterone administration in separate experiments, and the above protocol was repeated.

Micropuncture: Animal Preparation
Male, Sprague-Dawley rats, 250 to 350 g, were used. Methods for animal preparation were similar to those published previously (please see the online-only Data Supplement). TGF was determined by maximum changes of stop-flow pressure (Psd) when increasing tubular perfusion rate from 0 to 40 nL/min. First, the nephron was perfused with artificial tubular fluid containing vehicle for 3 to 5 minutes to get the initial measurement of Psd. Next, it was perfused for 10 minutes with vehicle or aldosterone (10^{-7} or 10^{-8} mol/L). In other experiments, to determine the role of NO in aldosterone-induced TGF alteration, l-NG-monomethyl arginine citrate (l-NMMA; Cayman Chemical, Ann Arbor, MI), 10^{-3}mol/L, was added into the perfusate, and the protocol above was repeated.

Laser Capture Microdissection and RT-PCR
Laser capture microdissection (LCM) and RT-PCR were used to isolate MD cells in Sprague-Dawley rats and to measure MR mRNA with methods that we have described previously (please see the online-only Data Supplement).

Immunofluorescence
The MR antibodies were developed and characterized as reported previously. An antibody for the Na^{+}-K^{+}·2Cl^{-} transporter (NKCC2) was a gift from Dr Pablo Ortiz at Henry Ford Hospital (Detroit, MI). Rat kidney sections were double stained with MR and NKCC2 antibodies and subsequent fluorescent secondary antibodies and observed under the Nikon microscope (please see the online-only Data Supplement).

Cell Culture and NO Measurement
Experiments were undertaken, using MMDD1 cells, an MD-like cell line (kindly provided by Dr J. Schnermann, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) in a manner similar to our previously described studies. The cells-permeable fluorescent NO indicator, 4,5-diaminofluorescein diacetate, 10 μmol/L, was used to measure NO before incubation and 15 minutes after incubation with aldosterone (10^{-8} mol/L; please see the online-only Data Supplement).

Statistical Analysis
Data were collected as repeated measures over time under different conditions. We tested only the effects of interest, using ANOVA for repeated measures and a post hoc Fisher least-significant difference test or a Student paired t test when appropriate. The changes were considered to be significant if P<0.05. Data are presented as mean±SEM.

Results

MR Expression in the MD
Figure 1 shows immunofluorescent results and positive staining for MR in the MD with intensity similar to that in thick ascending limbs but weaker than in distal tubules and medulary collecting ducts. We also used an NKCC2 antibody to mark MD and the thick ascending limbs. Note that MR is expressed in MD, as well as in thick ascending limbs and distal tubules, whereas NKCC2 is expressed in thick ascending limbs and MD but not in distal tubules.

To confirm MR expression in MD, we also used LCM to microdissect MD cells from frozen rat kidney sections and performed RT-PCR on the captured cells to measure mRNA expression. As shown in Figure 2A, the anatomic location and morphology of MD cells were identified with the LCM microscope before dissection. The cells were then thawed and combined with the membrane (Figure 2B), and cells were removed by lifting the membrane (Figure 2C). Figure 2D indicates that MR mRNA was expressed in microdissected MD cells. The negative control did not contain cDNA. Thus, we confirmed MR expression in the MD with 2 independent methods.

Aldosterone Blunts In Vitro TGF Measured With Microperfusion
To test the direct effect of aldosterone on TGF, we microdissected and perfused rabbit Af-Arts and glomeruli with the
To determine the role of MR in the acute effect of aldosterone on TGF, eplerenone (10⁻³ mol/L), a selective MR antagonist, was used. As shown in Figure 3C, eplerenone added in the tubular perfusate for 30 minutes and present during the rest of the experiment. When sodium concentration in tubular perfusate was increased from 10 to 80 mmol/L, the Af-Art diameter decreased from 18.9±0.4 to 16.1±0.7 μm. TGF, as shown in Figure 3D, was 2.8±0.7 μm. Then, the tubular perfusate was switched back to 10 mmol/L of NaCl, and aldosterone was added for 15 minutes. When we increased tubular NaCl to 80 mmol/L in the presence of aldosterone, the Af-Art diameter decreased from 18.9±0.7 to 16.5±0.3 μm. TGF was, thus, 2.4±0.5 μm (n=5). These data indicate that the effect of aldosterone on TGF was attenuated by MR antagonism, suggesting that the inhibitory effect of aldosterone on TGF was primarily mediated via MR activation.

**Aldosterone Blunts In Vivo TGF Measured by Micropuncture**

We performed micropuncture to test whether aldosterone affects the TGF response in vivo. The results of time control experiments are shown in Figure 4A (n=4 rats/7 tubules). When the tubular perfusion rate of vehicle was increased from 0 to 40 nL/min, Psf decreased from 38.9±1.3 to 30.1±1.1 mm Hg, and the change in Psf (ΔPsf), used as the TGF response, was 8.8±0.9 mm Hg. Then we reduced tubular perfusion and waited for the Psf to return to baseline, which was 38.2±1.3 mm Hg. When we increased tubular perfusion rate to 40 nL/min again, Psf decreased to 30.0±1.5 mm Hg. ΔPsf was 8.1±1.0 mm Hg. There was no significant difference between the 2 TGF responses, indicating that the TGF response is reversible and was a suitable control for the following experiments.

We tested whether aldosterone had any effect on TGF in vivo by adding aldosterone to the tubular perfusate and measuring TGF. When tubular perfusate was increased from 0 to 40 nL/min with vehicle, Psf decreased from 39.7±2.1 to 31.0±2.9 mm Hg. The ΔPsf was 9.1±1.0 mm Hg. Then we increased the concentration of aldosterone to 10⁻⁷ mol/L in the above experiments. As shown in Figure 4C, when perfused with vehicle only (control), Psf was reduced from 38.8±1.3 to 28.7±1.9 mm Hg, and ΔPsf was 10.1±1.4 mm Hg. In the presence of aldosterone, the Psf was reduced from 38.4±2.1 to 29.7±2.1 mm Hg. The ΔPsf was 9.0±0.9 mm Hg. There was no significant difference in TGF with and without aldosterone (Figure 4B; n=4 rats/7 tubules). Then we increased the concentration of aldosterone to 10⁻⁷ mol/L, in the above experiments. As shown in Figure 4C, when perfused with vehicle only (control), Psf was reduced from 38.8±1.3 to 28.7±1.9 mm Hg, and ΔPsf was 10.1±1.4 mm Hg. In the presence of aldosterone, the Psf was reduced from 39.3±1.6 to 31.5±1.6 mm Hg, and ΔPsf was 7.7±1.2 mm Hg (n=4 rats/9 tubules; P<0.05). Figure 4D shows a representative experiment demonstrating the changes of stop flow pressure responding to vehicle or aldosterone perfusion. Arrows indicate where Psf was measured. These data suggest that aldosterone blunted the TGF response in vivo.

To determine whether NO is involved in aldosterone-induced TGF inhibition, we used l-NMMA, a nonselective NO synthase (NOS) inhibitor in the lumen. First, l-NMMA
(10⁻³ mol/L) alone was tested in control experiments. When tubular perfusate was increased from 0 to 40 nL/min with l-NMMA, Psf decreased from 36.8±2.4 to 24.7±2.7 mm Hg, and \( \Delta P_{sf} \) was 12.1±1.4 mm Hg. In a repeat of control conditions, after Psf returned to baseline, tubular perfusion rate was increased from 0 to 40 nL/min, and Psf decreased from 36.4±2.3 to 22.9±3.1 mm Hg, \( \Delta P_{sf} \) was 13.6±1.6 mm Hg (Figure 5A; n=5 rats/8 tubules; \( P<0.05 \)). In the next group of experiments, aldosterone, 10⁻⁷ mol/L, was added to the tubular perfusate in the presence of l-NMMA. When perfused with l-NMMA alone, Psf decreased from 37.5±1.9 to 24.3±1.7 mm Hg, and \( \Delta P_{sf} \) was
13.2 ± 1.4 mm Hg. In the presence of aldosterone, Psf decreased from 37.5 ± 1.7 to 21.8 ± 1.8 mm Hg, and ΔPsf was 15.7 ± 1.0 mm Hg. The changes between the first and second TGF responses in the absence and presence of aldosterone were not significantly different. As shown in Figure 5B, the effect of aldosterone on TGF was totally blocked when NO was inhibited (n = 4 rats; n = 7 tubules). We also compared the difference between TGF response to aldosterone alone (Figure 4C) and aldosterone plus L-NMMA (Figure 5B). The difference was significant (P < 0.01). These data suggest that NO was involved in mediating the acute effect of aldosterone on TGF, because blockade of NOS totally prevented the attenuation of TGF by aldosterone.

Figure 4. Aldosterone blunts tubuloglomerular feedback (TGF) in vivo. In microperfusion study in Sprague-Dawley (SD) rats, maximum change of stop-flow pressure (ΔPsf) when increasing tubular flow from 0 to 40 nL/min was used to determine TGF. A, There was no difference of ΔPsf when TGF responses were induced and repeated with only vehicle. B, Aldosterone at 10^{-8} mol/L had no effect on TGF response. C, In the presence of aldosterone (10^{-7} mol/L) in tubular perfusate, ΔPsf decreased from 10.1 ± 1.4 to 7.7 ± 1.2 mm Hg (n = 4 rats/9 tubules; P = 0.05). Panel D shows a representative microperfusion experiment of TGF measurement induced by switching tubular perfusion rate and measured by change of Psf. Blood pressure was stable during measurement. Arrows indicate where Psf was measured.

Aldosterone Stimulates NO Production in Cultured MD Cells

To test whether aldosterone enhances NO generation by the MD cells, we loaded MMDD1 cells with 4,5-diaminofluorescein diacetate to measure NO generation with and without aldosterone. In basal conditions, the rate of NO generation was 40.4 ± 4.3 U/min. After adding aldosterone for 15 minutes, the rate of NO production increased significantly to 644.1 ± 118.5 U/min (Figure 5C; n = 16; P < 0.01 versus basal). In time control experiments only with vehicle, the rate of increase in NO generation was 45.9 ± 5.2 U/min at basal and 53.4 ± 6.1 U/min 15 minutes later (n = 11).

Discussion

A novel finding of the present study is that mRNA and protein for MR are expressed in the MD cells. Second, we found that aldosterone blunted the TGF response both in anesthetized rats in vivo and in microperfused JGA in vitro. Third, the MR antagonist eplerenone abolished aldosterone-induced TGF inhibition. Fourth, NOS inhibition restored the blunted TGF. Fifth, aldosterone markedly increased NO generation by MMDD1 cells. Taken together these data suggest that aldosterone attenuates TGF by a MR-mediated event resulting from release of NO by the MD.

Previous studies using autoradiographic methods and immunostaining have found MR in distal tubules, connecting and cortical collecting tubules, and in medullary and papillary collecting ducts.11,12 MR was also found in the thick ascending limb of the loop of Henle using RT-PCR and recently in glomeruli by immunostaining.14 Using highly specific anti-
citrate (L-NMMA), 10^{-3} \text{ mol/L}, was perfused into tubules to block TGF responses. In the present study, we used LCM to acquire MD cells and demonstrated mRNA for MR in the MD. These observations, when combined with our immunohistochemistry data, clearly indicate significant MR expression in MD cells.

Our results also indicate that aldosterone-mediated activation of MR in the MD has a functional role in altering TGF. Using in vitro microperfused JGA, as well as in vivo micropuncture experiments, we found that aldosterone attenuated TGF by \sim 50\%. This effect was completely reversed by blocking NO synthesis, indicating that NOS plays a primary role in TGF resetting by aldosterone. To our knowledge, there have been no previous reports of the direct effects of aldosterone on TGF. Only a few studies have been conducted to examine the effects of MR activation on renal hemodynamics. Arima et al^{15,16} showed that acute administration of aldosterone caused constriction in rabbit arterioles, and NOS inhibition further augmented this vasoconstriction. In contrast, Uhrenholt et al^{17} found in renal afferent arterioles a vasodilator effect of aldosterone that was abolished by blockade of NOS. Schmidt et al^{18} also found in human forearm vessels that aldosterone caused vasodilation and increased blood flow, but after administration of L-NMMA, forearm blood flow significantly decreased during aldosterone infusion. Thus, although there is still controversy concerning the vascular effects of aldosterone, the signaling pathways consistently point to NO, which is consistent with our observation that the rapid effect of aldosterone on TGF is mediated by NO.

To further test whether aldosterone enhances NO generation in the MD, we measured NO generation in MMDD1 cells with a fluorescent dye. We found that aldosterone significantly enhanced NO generation by MMDD1 cells. We reported recently that aldosterone also stimulates superoxide generation in MMDD1 cells. In that study we excluded the effect of NO by using l-arginine–free solutions. However, in the present study, l-arginine was present in solutions used for the experiments with MMDD1. Therefore, enzymes that generate both NO and superoxide should have been intact in the present study. The detection of either NO or superoxide should, therefore, reflect the net effect of the interaction between the NO and superoxide. A significant increase of NO was detected in MMDD1 cells stimulated by aldosterone, indicating that production of NO exceeded that of superoxide.

Our results also indicate that the effects of aldosterone to reduce TGF responses occur rapidly, within 10 minutes. When aldosterone functions through a genomic pathway, it couples with MR, and this product functions as a transcription factor. However, rapid nongenomic effects of aldosterone that do not require transcription or protein synthesis have also been reported in various tissues, such as the heart, colon, renal tubule, and vascular smooth muscle. As early as 1958, the rapid action of aldosterone on urinary electrolyte excre-
tion was reported to occur in 5 minutes.19 In rat cortical collecting tubules, an aldosterone-induced ion influx occurred within 30 minutes.20 A series of studies demonstrated that aldosterone acts within minutes to alter cellular pH and plasma membrane potassium conductance in various cell preparations by rapidly increasing net entry of Ca2+, activating membrane Na+/H+ exchanger, and modulating K+ channel activity.21–23 These effects were shown to be spironolactone insensitive and blocked by protein kinase C inhibitors.24 The involvement of extracellular signal–regulated kinase 1/2 in the rapid nongenomic action was shown in MDCK cells25 and in the renal medullary thick ascending limb of the loop of Henle.26

The concentration of aldosterone required for rapid nongenomic effects has varied from subnanomolar up to 10 nmol/L.27,28 In the present study, the rapid effect of aldosterone occurred within 10 minutes in microperfused JGA and in micropuncture studies, suggesting that a nongenomic pathway may be involved. For the in vivo micropuncture studies, a higher dose of aldosterone was required to alter TGF. The reason for this is not clear but may be attributed to the micropuncture technique used. For example, effective concentrations of NOS inhibitors needed to inhibit TGF in renal micropuncture studies29 are usually higher than used in JGA microperfusion.30 We cannot measure the actual concentration at the MD under current experimental settings but assume that the aldosterone concentration that actually reaches the MD cells is considerably less than the concentration in the proximal tubule when using micropuncture methods. Overall, our results suggest a novel mechanism by which aldosterone may influence GFR regulation through a nongenomic pathway.

In the present study we found that eplerenone completely blocked the effect of aldosterone on TGF, indicating that the effects are mediated through activation of MR. In agreement with our observations, the acute effects of aldosterone on small resistance mesenteric vessels31 and renal afferent arterioles32 are also mediated by activation of MR. However, it has also been reported that acute infusion of aldosterone had no effect on GFR both in humans33 and animals.34 These data do not necessarily contradict our findings in the present study. We found that aldosterone suppressed TGF and dilated AF-Art through the MR of the MD. Arima et al15,16 reported that aldosterone directly constricted the AF-Art. Therefore, the effect of acute injection of aldosterone may reflect the balance of effects on the MD, which would tend to inhibit TGF and raise GFR, and direct vasoconstrictor effects on AF-Art, which would tend to decrease GFR. The net acute effect of aldosterone could be unchanged GFR as a result of these offsetting effects. In secondary hyperaldosteronism, which is often associated with high levels of angiotensin II, which enhance TGF, the increased levels of aldosterone may serve to buffer the effect of angiotensin II on TGF and GFR.

**Perspectives**

Our observations provide a potential mechanism by which high levels of aldosterone may cause glomerular hyperfiltration, a risk factor for renal injury and chronic kidney disease, in patients with primary aldosteronism, as well as in obese subjects who have increased renal MR activation.2,34 Our results also indicate that the rapid effects of aldosterone on TGF are mediated through synthesis of NO at the MD and may provide a potential target for treating obesity hypertension and associated chronic kidney disease. Further studies are needed to elucidate the physiological and pathophysiological significance of this novel effect of aldosterone on TGF.

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**Disclosures**

None.

**References**

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Methods

All procedures and experiments were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. All chemicals were purchased from Sigma (St. Louis, MO) except as indicated.

Microperfusion

Isolation and microperfusion of the rabbit Af-Art and attached MD: We used methods similar to those we described previously to isolate and microperfuse the Af-Art and attached MD. Briefly, young male New Zealand white rabbits (1.5 to 2.0 kg) were anesthetized with sodium pentobarbital (40 mg/kg i.v.) and given an injection of heparin (500 U i.v.). The kidneys were removed and sliced along the corticomedullary axis. Slices were placed in ice-cold minimum essential medium (MEM; Gibco, Grand Island, NY) containing 5% bovine serum albumin and dissected under a stereomicroscope (model SMZ 1500; Nikon). From each rabbit, a single superficial Af-Art and its intact glomerulus were microdissected together with adherent tubular segments consisting of portions of the thick ascending limb of the loop of Henle, MD, and the early distal tubule. Using a micropipette, the microdissected complex was transferred to a temperature-regulated chamber mounted on an inverted microscope (Eclipse Ti; Nikon) with Hoffman modulation. Both the Af-Art and the end of either the distal tubule or thick ascending limb were cannulated with an array of glass pipettes. The Af-Art was perfused with MEM, and intraluminal pressure was maintained at 60 mm Hg throughout the experiment. The MD was perfused with physiologic saline consisting of (in mmol/L) 10 HEPES; 1.0 CaCO3; 0.5 K2HPO4; 4.0 KHCO3; 1.2 MgSO4; 5.5 glucose; 0.5 Na acetate; 0.5 Na lactate, L-arginine 0.5; and either 80 NaCl (high NaCl) or 10 NaCl (low NaCl). The pH of the solution was 7.4. The perfusion bath was exchanged continuously at a rate of 1 mL/min. Microdissections were completed within 60 minutes at 8°C, and the samples were then transferred into the bath and gradually warmed to 37°C for the rest of the experiment. A 30-minute equilibration period was allowed before taking any measurements. The imaging system consisted of a microscope (Eclipse Ti; Nikon), digital charge-coupled device camera (CoolSnap; Photometrics), xenon light (LB-LS/30; Shutter Instruments), and optical filter changer (Lambda 10–3; Shutter Instruments). Images were displayed and analyzed with NIS-Elements imaging software (Nikon).

Experimental protocols: After the 30-minute equilibration period, the MD perfusate was switched from low to high NaCl, and luminal diameter of the Af-Art was observed for 5 minutes. We used the average change in diameter of the Af-Art as our control TGF response. Then the MD perfusate was switched back to low NaCl. To study the effect of aldosterone on regulation of the TGF response in vitro, aldosterone (10^-8 mol/L) was added to the tubular perfusate for 15 min and a second TGF response was measured. To study if the aldosterone-induced TGF alteration could be inhibited by MR blockade, in separate experiments, a selective MR antagonist, eplerenone, 10^-5 mol/L, was added to the tubular perfusate. The above protocol was repeated.
Micropuncture

Animal preparation: Methods for animal preparation were similar to those previously published2-4, with modification. Male Sprague-Dawley (SD) rats (Harlan Laboratories, Indianapolis, IN) weighing between 250 to 350 g were anaesthetized by inactin (I.P. 70mg/Kg) and ketamine (I.M., 50mg/Kg). Body temperature was maintained at approximately 36.5°C by a heated operating table controlled by feed-back from a rectal thermometer (Vestavia Scientific, Birmingham AL). A tracheal cannula was inserted with PE240 tubing for unrestricted respiration. The left femoral artery was catheterized for continuous recording of blood pressure by a pressure transducer. The jugular vein was catheterized for sustaining infusion of a 0.9%NaCl saline with 1% bovine serum albumin at 0.5 ml/hr 100g BW3. The bladder was catheterized via a suprapubic incision along the linea. The left kidney was exposed by a flank incision, dissected free from its perirenal attachments, laid in a kidney cup, and embedded with 4% agar in saline. The renal surface was bathed in saline. After an equilibration period of 30-45 min, a random proximal tubular segment was punctured with a micropipette (o.d., 6-8 µm) filled with artificial tubular fluid (ATF) (containing in mmol/L: 4 NaHCO₃, 5 KCl, 2 CaCl₂, 7 Urea, 2 MgCl₂, 128 NaCl, pH 7.4) and stained with 0.1% fast green. Superficial segments of the proximal convoluted tubule were identified by injection of fast green solution. A grease pipette (o.d., 12µm) was then inserted on the same site and flow was blocked by injection of a grease block. A perfusion pipette (o.d., 6-8 ,µm) containing artificial tubular fluid plus dye was inserted into the last segment of the late proximal tubule downstream from the grease block and connected to a nanoliter microperfusion pump (Vestavia Scientific, Birmingham, AL). A pressure pipette (o.d., 2-5µm), filled with 2mol/L KCl and connected to a micropressure system (model 900A, World Precision Instruments, Sarasota, FL), was inserted upstream from the grease block to measure proximal stop-flow pressure (Psf). When the perfusion rate was switched from 0 nL/min to 40 nL/min, delta change of Psf was recorded as an indicator of TGF. Psf and mean arterial pressure (MAP) were recorded and displayed by a PowerLab data acquisition system (ADInstruments, Colorado Springs, CO). At the end of each experiment, an arterial blood sample was taken with a heparinized capillary tube to measure hematocrit.

Experiment protocols: In the same nephron, TGF responses were measured twice by switching tubular perfusion rate repeatedly. First, the nephron was perfused with ATF containing vehicle for 3-5 min to get the initial determination of Psf. Next, it was perfused for 10 min with vehicle or aldosterone (10⁻⁷ mol/L or 10⁻⁸ mol/L). In other experiments, to determine the role of nitric oxide (NO) in aldosterone-induced TGF alteration, L-NMMA (L-NG-monomethyl arginine citrate, Cayman Chemical, Ann Arbor, MI) was added into the perfusate and the protocol above was repeated.

Laser capture microdissection and RT-PCR

Laser capture microdissection(LCM) was used to isolate rat MD cells with methods we have described previously5. LCM is a technique based on the adherence of visually selected cells to a thermoplastic membrane, which is focally melted by triggering a low-energy infrared laser pulse. The melted membrane forms a composite with the selected tissue area that is removed by lifting off the membrane6. Kidneys from SD rats were removed and snap-frozen in Optimal Cutting Temperature Medium. Eight-micrometer-
thick frozen sections were obtained, and then stained and dehydrated using an Arcturus Histogene frozen section staining kit (Life Technologies, Carlsbad, CA). The MD cells were dissected with an Arcturus Laser Capture Microdissection System (Model Veritas). At least 20 MD cells were required to extract enough RNA for PCR measurement.

Total RNA was isolated using an Arcturus PicoPure RNA isolation kit and 10 µl of this RNA were amplified by Message Sensor RT kit. PCR was performed in a BioRad thermal cycler (Bio-Rad, Hercules, CA). Negative controls were performed by omitting cDNA template from the PCR amplification. Quality control experiments were performed by testing the mRNA expression of nNOS and eNOS, as shown in Figure S1. MR primers: 5’-GCCCGGCAAATCTCAACAACTCAA-3’ (forward primer) and 5’-TTAGGGAAAGGAACGTCGTGAGCA-3’ (reverse primer). GAPDH served as a "housekeeping" gene. The mixed samples were heated to 95°C for 5 min and then cycled for 40 at 94°C 30sec—59°C 20sec—72°C 30sec. Final extension was 8 min at 72°C. Complimentary DNA was amplified simultaneously for all tissues, electrophoresed on a 1.5% agarose gel, and stained with ethidium bromide. Images were captured using a VersaDoc image analysis system (Bio-Rad, Hercules, CA).

Immunofluorescence

Kidneys were collected from anesthetized rats, and perfused with saline, followed by Streck Tissue Fixative (STF; Streck, Inc., Omaha, NE) as previously described. The tissues were further fixed in STF overnight, and then embedded in paraffin. Six-micrometer sections were cut, deparaffinized, treated with 0.1% phenylhydrazine for 30 min to inhibit peroxidases, then blocked with with 0.1 M Tris, 2.5% dry milk, 5% normal goat serum, and 0.1% Triton X-100 for 1 h. The slides were then incubated overnight with the MR antibodies that were described and characterized previously and NKCC2 antibody (a gift from Dr. Pablo Ortiz, Henry Ford Hospital, Detroit MI) in the same buffer. After washing, the sections were incubated for 1 h with ImmPress Goat anti-Mouse(Rat absorbed, Vector Laboratories, Burlingame, CA) and Alexa-594 Goat anti-Rabbit IgG(Invitrogen, Carlsband, CA), washed, and incubated with Biotin-Tyramine in imidazole buffer for 30 min. Then slides were incubated with Avidin NeutrAvidin Alexa-488 (Invitrogen, Carlsband, CA) for 30min. Negative controls included incubation with no primary antibody and with primary antibody preincubated with an excess of immunizing peptide. All slides were mounted with anti-fade medium. Images were captured in an imaging system (Nikon Eclipse Ti).

Cell culture and NO measurement

Experiments were undertaken in MMDD1 cells, a MD-like cell line (kindly provided by Dr. J. Schnermann, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Similar to our previously reported protocols, MMDD1 cells at passages 20–25 were cultured on a small piece of sterilized coverglass in DMEM nutrient mixture-Ham's F-12 (DMEM/F12, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100µg/ml streptomycin) and incubated in a humidified atmosphere of 95% room air-5% CO2 at 37°C. When cells reached 70%-80% of confluence, they were loaded with 10 µmol/L 4,5-diaminofluorescein diacetate (DAF-2 DA), a cell-permeable fluorescent NO indicator, in 0.5% dimethyl sulfoxide (DMSO) for
30 minutes. Then, they were transferred to a glass chamber mounted on the inverted microscope, with the temperature maintained at 37°C. The chamber was perfused with DMEM/F12 and was exchanged continuously at a rate of 1 ml/min. DAF-2 DA was excited at 490 nm and emitted fluorescence is recorded at 510 to 550 nm. Square-shaped regions of interest (ROIs) were set inside the cytoplasm of the cells and mean intensity of ROIs recorded every 5 seconds and corrected for background. The rate of increase in intensity was calculated (increase of units/min). After a 20-min equilibration period, NO was measured for 5 min. Then aldosterone (10⁻⁸M) was added into bath for 10 min, NO was measured again for 5 min. Time control experiments were performed using the same protocol without aldosterone treatment.

Statistical analysis

Data were collected as repeated measures over time under different conditions. We tested only the effects of interest, using analysis of variance (ANOVA) for repeated measures and a post-hoc Fisher LSD test or a paired T test when appropriate. The changes were considered to be significant if P<0.05. Data are presented as mean ± SEM.

Reference List


**Figure S1. Quality control of LCM sample.** A positive nNOS was taken as a marker of the macula densa; and a negative eNOS indicating no contamination by surrounding cells.