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Oxidative status in the macula densa modulates tubuloglomerular feedback responsiveness in Ang II-induced hypertension

Jiangping Song^{1,2}, Yan Lu^{2,3}, En Yin Lai⁴, Jin Wei², Lei Wang², Kiran Chandrashekar³, Shaohui Wang², Chunyu Shen², Luis A. Juncos^{2,3}, and Ruisheng Liu^{2,3,*}

¹ State Key Laboratory of Cardiovascular Disease, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, 167A Beilishi Road, Xi Cheng District, Beijing, 100037, China

² Department of Physiology & Biophysics, University of Mississippi Medical Center, 2500 N. State St., Jackson MS 39216, USA

³ Division of Nephrology, Department of Medicine, University of Mississippi Medical Center, 2500 N. State St., Jackson MS 39216, USA

⁴ Department of Physiology Zhejiang University, Hanzhou, China

Abstract

Aim—Tubuloglomerular feedback (TGF) is an important mechanism in control of signal nephron glomerular filtration rate. The oxidative stress in the macula densa, primarily determined by the interactions between nitric oxide (NO) and superoxide (O_2^-), is essential in maintaining the TGF responsiveness. However few studies examining the interactions between and amount of NO and O_2^- generated by the macula densa during normal and hypertensive states.

Methods—In this study, we used isolated perfused juxtaglomerular apparatus to directly measure the amount and also studied the interactions between NO and O_2^- in macula densa in both physiological and slow pressor Angiotensin II (Ang II) induced hypertensive mice.

Results—We found that slow pressor Ang II at a dose of 600ng/kg/min for two weeks increased mean arterial pressure by 26.1±5.7 mmHg. TGF response increased from 3.4±0.2 μ m in control to 5.2±0.2 μ m in hypertensive mice. We first measured O_2^- generation by the macula densa and found it was undetectable in control mice. However, O_2^- generation by the macula densa increased to 21.4 ± 2.5 unit/min in Ang II-induced hypertensive mice. Then we measured NO generation and found that NO generation by the macula densa was 138.5 ± 9.3 unit/min in control mice. The NO was undetectable in the macula densa in hypertensive mice infused with Ang II.

Conclusions—Under physiological conditions, TGF response is mainly controlled by the NO generated in the macula densa; in Ang II induced hypertension, the TGF response is mainly controlled by the O_2^- generated by the macula densa.

*Corresponding author: Ruisheng Liu, MD, PhD Department of Physiology & Biophysics University of Mississippi Medical Center 2500 N. State St., Jackson MS 39216 Phone: 601-815-1401 FAX: 601-984-1817 rliu@umc.edu.

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Keywords

macula densa; tubuloglomerular feedback; hypertension; oxidative stress

INTRODUCTION

The macula densa plaque consists of a group of specialized epithelial cells located in the distal segment of the thick ascending limb of the nephron which serve as luminal sensors of sodium chloride (NaCl) concentration. An increase in the NaCl delivery to the macula densa promotes release of adenosine and/or ATP, leading to afferent arteriolar constriction and decrease in the single nephron glomerular filtration rate (SNGFR); this process is termed as the tubuloglomerular feedback (TGF) response (Briggs & Schnermann 1986; Ollerstam et al. 1997; Welch et al. 2000; Ren et al. 2000). TGF may help to maintain the extracellular fluid volume by preserving the delicate balance between the glomerular filtration and tubular reabsorption rate (Briggs & Schnermann 1986; Welch & Wilcox 1990; Schnermann & Briggs 1992). TGF does this by establishing a negative feedback through which any change in NaCl delivery to the macula densa induces a reciprocal change in SNGFR and tubular flow, thereby preventing acute fluctuations of flow and NaCl delivery in the distal nephron. Hence, TGF limits urinary volume and sodium excretion, thereby helping maintain the salt and water homeostasis. Several in-vivo and in-vitro models of hypertension and sodium retaining states such as cirrhosis and acute renal failure have been found to have an augmented TGF response (Welch et al. 1999; Welch et al. 2000; Persson et al. 2000; Blantz et al. 2002; Carlstrom et al. 2009a; Carlstrom et al. 2013), implying that abnormal TGF responses may have a potential role in these pathophysiological conditions.

Several factors regulate TGF responsiveness, including angiotensin II (Welch & Wilcox 1990; Ren et al. 2002c; Vallon et al. 2004), adenosine (Sun et al. 2001; Ren et al. 2002a; Li et al. 2013; Sallstrom et al. 2013), arachidonic acid metabolites (Zou et al. 1994a; Zou et al. 1994b; Imig et al. 1996; Imig 2000), ATP (Inscho et al. 1992; Nishiyama et al. 2001; Nishiyama & Navar 2002; Ren et al. 2004), atrial natriuretic factor (Huang & Cogan 1987), superoxide (O_2^-) (Welch et al. 2000; Ren et al. 2002b; Liu et al. 2004b) and nitric oxide (NO) (Ollerstam et al. 1997; Ichihara et al. 1998; Ichihara & Navar 1999; Welch et al. 2000; Ren et al. 2000; Liu et al. 2004a). Among these factors, oxidative stress in the macula densa (which primarily depends on the interaction between NO and O_2^-), is crucial in controlling TGF responses both in normal and hypertensive animals models (Welch et al. 2000; Araujo & Welch 2006; Fu et al. 2012; Zhang et al. 2014). Several studies have demonstrated that both the NO and O_2^- generated by the macula densa are enhanced during TGF and in turn regulate the TGF responsiveness (Liu et al. 2002; Kovács et al. 2003; Liu et al. 2004a; Liu et al. 2004b; Liu et al. 2008; Zhang et al. 2009). Additionally, O_2^- is vital in regulating TGF responses during pathophysiological situations such as hypertension, diabetes and high salt intake (Welch & Wilcox 1997; Welch et al. 1999; Welch et al. 2006; Zhang et al. 2014). However, despite their obvious significance, to date most of the studies have only targeted the enzymes producing either NO or O_2^- with antagonists or genetic approaches. There has been a lack of studies examining the interactions between and also the amount of NO and O_2^- being generated by the macula densa during physiological conditions and the possible

alterations to these molecules during hypertension. Hence, in this study, we hypothesized that interactions between NO and O_2^- determines the oxidative stress in the macula densa and regulates TGF responsiveness. We directly measured the NO and O_2^- generation in the macula densa with fluorescent dyes in isolated perfused juxtaglomerular apparatus and also compared their levels in normal and hypertensive states.

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.

Telemetry transmitter implantation

As described previously (Zhang et al. 2014), C57BL/6 mice (18-20 g, Harlan) were anesthetized with inhaled isoflurane (Butler chemicals, UK). A small incision was made in the middle of neck and a telemetry transmitter (PA-C10, Data Sciences International) was inserted under sterile conditions. The catheter of the transmitter was placed in the left carotid artery and advanced down to the aortic arch. The body of the transmitter was placed subcutaneously in the right ventral flank of the animal. The mice were then allowed to recover for ten days.

Minipump Insertion

Osmotic minipumps (Alzet Corp) were filled with either slow-pressor dose of Angiotensin II (Ang II;600ng/kg/min) or saline (control) previously described (Zhang et al. 2014). On the day of surgery, the animals which had ten days to recover from the telemetry transmitter implantation were again anesthetized with isoflurane. Under sterile conditions, a small incision was made in the mid-scapular area on the animal's back. Using a hemostat, the subcutaneous tissue was separated and spread so as to create a small pouch in which the minipump was inserted. Following insertion, the wound was sutured and the animals were allowed to recover for a day. Measurements of the mean arterial pressure (MAP) were started on the second day after minipump implantation at 10 minute intervals. All in vitro experiments using hypertensive mice were performed at day 14 after minipump insertion.

Isolation and microperfusion of the mouse Af-Art and attached macula densa and measurement of TGF

The Afferent-arteriole (Af-Art) and attached macula densa were isolated and microperfused using previously described techniques (Liu et al. 2004b; Fu et al. 2012). Briefly, mice (18 to 20 g) were anesthetized with inhaled isoflurane. The kidneys were removed and sliced along the corticomedullary axis. These slices were then placed in ice-cold minimum essential medium (MEM; Gibco, Grand Island, NY) containing 5% bovine serum albumin and dissected under a stereo-microscope (model SMZ 1500; Nikon). 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, macula densa, and 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

Hoffmann 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 then perfused with MEM, and the intraluminal pressure was measured with a pressure pipette and maintained at 60 mm Hg throughout the experiment. The macula densa 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; and either 80 (high NaCl) or 10 NaCl (low NaCl). The pH of the solution was 7.4. The bath was exchanged continuously at a rate of 1 mL/min. Microdissection was completed within 60 minutes at 8°C, the samples were then transferred into the bath which was gradually warmed to 37°C for the rest of the experiment. After the 30-minute equilibration period, the macula densa perfusate was switched from 10 to 80 mmol/L NaCl, and luminal diameter of the Af-Art was observed for 5 minutes. The change in the diameter of the Af-Art was considered as the TGF response. 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).

Measurement of O₂⁻ and NO with fluorescent dyes in isolated perfused macula densa

The macula densa with its attached glomerulus was isolated and micro-perfused using previously described techniques (Liu & Persson 2004; Liu et al. 2007; Zhang et al. 2009; Lu et al. 2010). Briefly, a single superficial intact glomerulus was microdissected together with adherent tubular segments consisting of portions of the TAL, macula densa and early distal tubule. The TAL was cannulated with an array of glass pipettes. Another pipette was used to hold and stabilize the glomerulus. The sample was arranged so that each macula densa cell could be clearly visualized on the edge of the glomerulus.

A O₂⁻ sensitive fluorescent dye, dihydroethidium, was used to detect O₂⁻ levels (Fu et al. 2010; Zhang et al. 2014). Briefly, once the TAL was perfused, macula densa cells were loaded with 10 μM dihydroethidium in 0.1% dimethyl sulfoxide (DMSO) plus 0.1% pluronic acid from the lumen for 30 minutes then washed for 20 minutes. The loaded macula densa cells were exposed to 380 and 490 nm light to excite dihydroethidium and oxyethidium, respectively. Emitted fluorescence from dihydroethidium was recorded using a 420 nm dichroic mirror with a 460/50 nm bandpass filter; for oxyethidium we used a 565 nm dichroic mirror with a 605/55 nm bandpass filter. Square-shaped regions of interest were set inside the cytoplasm of macula densa cells and their mean intensity recorded every 5 seconds for 2 minutes. We recorded and calculated the rate of the changes for oxyethidium/dihydroxyethidium as an indicator of O₂⁻ production.

A NO-sensitive fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA) was used to detect NO production in macula densa (Liu et al. 2004a; Lu et al. 2010). The macula densa cells were loaded with 10 μM DAF-2 DA in 0.5% DMSO plus 0.1% pluronic acid from the tubular lumen for 30 to 40 minutes, then washed for 10 minutes. DAF-2 was excited at 490 nm with a xenon arc light, and the emitted fluorescence was recorded at wavelengths of 510 to 550 nm. Square-shaped regions of interest were set inside the cytoplasm of macula densa cells and mean intensity within the regions of interest was recorded every 5 seconds for 2

minutes. Relative changes in DAF-2 fluorescence intensity were recorded. We calculated the rate of the changes for DAF-2 intensity as an indicator of NO production. In the experiments using inhibitors, we added inhibitors in tubular lumen and bath for 15 minutes before measurement.

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 Student's paired t-test when appropriate. The changes were considered to be significant if $p < 0.05$. Data are presented as mean $\pm$ SEM.

RESULTS

Hypertension induced with slow-pressor Ang II infusion in mice

C57BL/6 mice were infused with slow pressor Ang II at a dose of 600ng/kg/min for two weeks and the blood pressure was measured using telemetry. The MAP gradually increased from a basal level of 101.4 ± 3.7 mmHg to reach a peak of 127.5 ± 8.2 mmHg on days 10-14 of the infusion ($p < 0.01$; $n = 8$). The MAP of control mice infused with saline did not vary significantly (basal MAP changed from 99.4 ± 4.3 to 103.7 ± 5.6 mmHg at the end of 2 weeks of infusion, respectively; $n = 11$, Fig 1).

TGF response in hypertensive mice induced with slow-pressor Ang II

TGF response was measured in isolated perfused JGAs from the hypertensive mice induced with slow-pressor Ang II and compared with controls. In mice without infusion of Ang II or saline, the diameter of the Af-Art was 16.3 ± 0.8 μ m when the tubule was perfused with a 10 mmol/L NaCl. After we switched the tubular NaCl concentration to 80 mmol/L, the Af-Art constricted to 12.9 ± 0.9 μ m. In this setting, the TGF response (as indicated by the delta changes of the A-Art diameter) was 3.4 ± 0.2 μ m ($n = 6$; Fig 2). In the mice infused with saline, when we increased tubular NaCl concentration from 10 to 80 mmol/L in the isolated perfused JGAs, Af-Art constricted from 17.1 ± 0.7 to 13.3 ± 0.6 μ m. TGF response was 3.8 ± 0.3 μ m. ($n = 6$; Fig 2). In the AngII infused hypertensive mice, when the tubular NaCl concentration was increased from 10 to 80 mmol/L in the isolated perfused JGAs, Af-Art constricted from 15.8 ± 0.7 to 10.6 ± 0.9 μ m. TGF response was 5.2 ± 0.2 μ m ($p < 0.01$ vs. Basal and saline-infused samples; $n = 6$; Fig 2). These data indicate that TGF responses were enhanced in slow-pressor AngII induced hypertensive mice.

O₂⁻ generation by the macula densa in Ang II-induced hypertensive mice

First we determined if the O₂⁻ generated by the macula densa cells increased following Ang II infusion. For this, we isolated and perfused the JGA and measured the O₂⁻ generated using dihydroethidium in mice infused with Ang II or saline for 2 weeks and compared them to basal values (Fig 3). The macula densa was perfused with an 80 mmol/L NaCl solution. O₂⁻ generation by the macula densa was undetectable in mice infused with saline and at basal conditions. However, O₂⁻ generation by the macula densa increased to 21.4 ± 2.5 unit/min in Ang II-induced hypertensive mice ($p < 0.01$, $n = 7$, Fig 4). These data suggest

that in control mice, the macula densa generated a greater amount of NO compared to O_2^- ; in hypertensive mice, O_2^- generation by the macula densa significantly increased.

Next, in order to determine the O_2^- generation by the macula densa cells without any interaction from NO, we repeated the above protocols in the presence of a non-selective NOS inhibitor, L-NAME (10^{-4} mol/L) in tubular lumen and bath in separate experiments. The macula densa was perfused with an 80 mM NaCl solution. Under basal conditions the O_2^- generation by the macula densa was 12.3 ± 1.1 unit/min. In saline infused mice, it was 14.7 ± 2.0 unit/min. In the slow-pressor Ang II infused hypertensive mice, the O_2^- generation by the macula densa increased to 45.3 ± 3.7 unit/min ($p < 0.01$, $n=5$, Fig 5). These data indicate that there is a significant increase in O_2^- generation by the macula densa in Ang II-hypertensive mice.

Finally, in order to establish whether the fluorescent signals measured were actually from O_2^- , we repeated the above protocols in normal control mice in the presence of L-NAME (10^{-4} mol/L) and a O_2^- scavenger, tempol (10^{-4} mol/L) in the bath and lumen in separate experiments. The macula densa was perfused with an 80 mmol/L NaCl solution. O_2^- generation was undetectable in the macula densa, indicating that fluorescent signals we measured were selective for O_2^- ($n=5$).

NO generation by the macula densa in Ang II-induced hypertensive mice

To determine whether NO generation by the macula densa increased in hypertensive mice, we measured NO using DAF-2 DA in isolated perfused JGAs in mice infused with Ang II. The macula densa was perfused with an 80 mmol/L NaCl solution. NO generation by the macula densa was 138.5 ± 9.3 unit/min in mice at basal state (without infusion of Ang II or saline). In mice infused with saline, NO generation by the macula densa was 143.9 ± 10.5 unit/min. However, the NO was undetectable in the macula densa in hypertensive mice infused with Ang II. ($p < 0.01$, $n=7$, Fig 6), indicating that in control mice, a greater amount of NO was generated compared to O_2^- by the macula densa; in hypertensive mice, there is more O_2^- generation by the macula densa than that of NO.

To determine the NO generated by the macula densa cells without any interaction from O_2^- , we repeated the above protocols in the presence of an O_2^- scavenger, tempol (10^{-4} mol/L) in the bath and lumen in separate experiments. As before, the macula densa was perfused with an 80 mM NaCl solution. NO generation by the macula densa was 168.4 ± 12.4 units/min in mice under basal conditions (without infusion of Ang II or saline). In mice infused with saline, NO generation by the macula densa was 172.6 ± 13.8 units/min. In the Ang II hypertensive mice, NO generation by the macula densa was 215.1 ± 14.9 units/min ($p < 0.01$, $n=6$, Fig 7). These data indicate that Ang II infusion enhances the NO generated by the macula densa.

Next, in order to determine the source of the NO being generated, we repeated above protocols in the presence of tempol (10^{-4} mol/L) and a selective NOS1 inhibitor 7-nitroindazole (7-NI, 10^{-4} mol/L) in the bath and lumen in normal control mice in separate experiments. The macula densa was perfused with an 80 mmol/L NaCl solution. Under these

conditions, NO generation was undetectable, indicating that the source of the macula densa NO generation was from NOS1 (n =4).

DISCUSSION

In this study we examined the interaction between NO and O_2^- generated by the macula densa and their role in the TGF response, under physiological and hypertensive states. The most important and novel findings are; that the interactions between NO and O_2^- in the macula densa regulates responsiveness of TGF; in addition these interactions vary in normotensive and hypertensive animals. In normotensive mice, there is a greater generation of NO compared to O_2^- by the macula densa and the NO dominates in controlling the TGF response. In contrast however, in Ang II-induced hypertension, O_2^- generation by the macula densa is greater than that of NO and the O_2^- becomes dominant in controlling the TGF response.

The slow-pressor Ang II-induced hypertension, which we have used in our present study is well recognized to be a good prototype of human hypertension and has been found to induce a gradual and sustained elevation in blood pressure over a period of time (Lever 1993; Rajagopalan et al. 1996; Reckelhoff et al. 2000). This slow-pressor Ang II induces oxidative stress, salt-sensitive hypertension, increases pro-inflammatory factors and leads to progressive renal injury (Cassis et al. 2009; Capone et al. 2011). The doses needed to cause these slow-pressor Ang II-induced changes vary based on route and species of animals. For example, previous studies have indicated that the changes induced by slow pressor doses of AngII in Sprague Dawley rats are generally stable and consistent (Chandrashekar et al. 2012). However, the changes induced in mice by such a dose are inconsistent; indeed the doses of slow pressor AngII needed to induce such changes in blood pressure and renal changes have varied considerably. The reasons are not clear, and may be due to variable release rates of the different models of minipumps or due to the varying responses of mice themselves. In mice, the slow-pressor dose fluctuates anywhere from 400-1000ng/kg/min in published reports (Daugherty et al. 2000; Brancaccio et al. 2003; Welch et al. 2006; Aragon et al. 2008; Cassis et al. 2009; Capone et al. 2011; Gao et al. 2011). In our hands, we found that Ang II at a dose of 600 ng/min/kg consistently elevated MAP by about 20 mmHg in 2 weeks (Zhang et al. 2014).

We found that TGF response was enhanced in isolated perfused JGAs from Ang II-induced hypertensive mice. Similarly, Dr. Welch's lab recently reported that slow-pressor Ang II infusion increased TGF response *in vivo* as measured by micropuncture (Araujo & Welch 2010). In our *in vitro* preparation, we have excluded all the confounding factors such as circulating Ang II and sympathetic activity. Hence, the observed enhanced TGF response should be due to the intrinsic changes in the macula densa cells and Af-Arts. It was not surprising and supported by our recent findings that both expression and activities of NOX2 and NOX4 in the macula densa were enhanced during Ang II-induced hypertension (Zhang et al. 2014). Enhanced oxidative stress in the macula densa augments TGF response (Wilcox & Welch 2000; Liu et al. 2004b).

We have reported previously that during TGF when we increased NaCl concentration in the macula densa from 10 to 80 mmol/L, both NO (Liu et al. 2002; Liu et al. 2004a; Liu & Persson 2005) and O_2^- (Liu et al. 2008) generation by the macula densa increased. However, their interaction at physiological and hypertensive conditions have not yet been clarified. The net effect between NO and O_2^- generated by the macula densa is vital in modulating the TGF responsiveness (Wilcox et al. 1998; Welch et al. 2000; Liu et al. 2004a; Liu et al. 2004b). In our present study, we performed a series of experiments to examine the interaction between NO and O_2^- generated by the macula densa during TGF, by directly measuring the concentration of NO and O_2^- with fluorescent dyes. We first measured the amount of O_2^- in normal mice and could not detect any signals for O_2^- , implying that the O_2^- was perhaps being scavenged by NO. To confirm this, we repeated the experiment in the presence of a NOS inhibitor to exclude the interaction between NO and O_2^- . We found a constant O_2^- generation by the macula densa in normal mice when the NO generation was inhibited, which was similar to our previous findings (Liu et al. 2007; Zhang et al. 2009). We next measured the amount of NO generated by the macula densa in normal mice and found a constant NO generation. The NO we measured is the net result of the interaction between NO and O_2^- in the macula densa. It also indicated under physiological conditions, the macula densa generates greater amounts of NO compared to O_2^- . To further confirm this, we repeated the experiments in the presence of the O_2^- scavenger, tempol. We found that NO generation increased by about 20% compared with experiments without tempol.

We have previously reported that Ang II activates NOS1 in the macula densa and enhances NO generation via AT1 receptors (Liu & Persson 2004). Moreover, we have also recently published that Ang II enhances O_2^- via increasing activity and expression of NOX2 and/or NOX4 isoforms of NAD(P)H oxidase in the macula densa. A series of elegant studies have been conducted regarding the function of oxidative stress in the macula densa in control of TGF and renal hemodynamics in Ang II infused animals (Wilcox & Welch 2000; Welch et al. 2006; Nouri et al. 2007; Carlstrom et al. 2009b; Wang et al. 2010; Carlstrom et al. 2010; Gao et al. 2011; Lai et al. 2012). In our present study we have directly measured the generation of NO and O_2^- and their interaction in the macula densa in Ang II-induced hypertensive mice. We found a constant O_2^- generation in the macula densa in Ang II-induced hypertensive mice, indicating more O_2^- was generated than NO in the macula densa. In the presence of NO inhibition, O_2^- generated increased by 1 fold compared with experiments without NO inhibition. Also, the O_2^- generation increased by more than 3 folds compared with experiments without hypertension. When we measured NO generation in the macula densa in Ang II-induced hypertensive mice, we did not detect any signals for NO, indicating that the NO was being scavenged by O_2^- generated in the macula densa. To confirm this, we repeated the experiments in the presence of the O_2^- scavenger, tempol. We found a constant NO generation in the macula densa in hypertensive mice and the NO generation increased by 25%. These data indicate that in Ang II-induced hypertensive mice, generation of both NO and O_2^- is increased by the macula densa. However, greater amount of O_2^- is generated compared to NO thereby contributing to the enhanced TGF response in Ang II-induced hypertension. These data also indicate that NAD(P)H oxidase is more sensitive to Ang II stimulation than NOS1 in the macula densa and plays key role in pathophysiological conditions such as Ang II-induced hypertension. However, we need to be

aware the limitations and pitfalls using fluorescent dyes, such as photobleaching, fluorophore saturation, background offsets and detector saturation to minimize the artifact (Brown 2007; Wardman 2007).

In summary, we examined the generation and interaction between NO and O_2^- generated by the macula densa both in physiological and hypertensive conditions. We found that under normal conditions, the generation of both NO and O_2^- by the macula densa increased during TGF. Under physiological conditions, NO dominates the oxidative stress in the macula densa. However, in Ang II-induced hypertension, while the macula densa increased generations of both NO and O_2^- , the amount of O_2^- generated is greater than that of NO. Hence, during Ang II chronic infusion, the O_2^- dominates the oxidative stress in the macula densa, which enhances TGF responsiveness and may contribute to the development of hypertension.

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Figure 1. A slow pressor dose of Angiotensin II infusion induced hypertension

Saline (control; n=11) or slow pressor dose of Angiotensin II (Ang II) at 600 ng/min/kg (n=8) was infused for 2 weeks in C57BL/6 mice. In the Ang II group the mean arterial pressure (MAP) increased from 101.4±3.7 mmHg to 127.5±8.2 mmHg after two weeks. In the control group the blood pressure did not vary significantly (MAP was 99.4±4.3 and 103.7±5.6 mmHg at basal and 2 weeks of infusion) (* P< 0.01 vs saline-infusion and basal)

Figure 2. Slow pressor Ang II induced Tubuloglomerular Feedback (TGF) response

The delta changes of the diameters of the afferent arterioles (Af-Art) are taken as an indicator of the TGF response. In mice without Ang II or saline infusion (basal state), the diameter of the Af-Art was $16.3 \pm 0.8 \mu\text{m}$ when the tubule was perfused with a 10 mmol/L sodium chloride (NaCl). When the tubular NaCl concentration was switched to 80 mmol/L, the Af-Art constricted to $12.9 \pm 0.9 \mu\text{m}$ (Fig 2A). The delta changes/ TGF response was $3.4 \pm 0.2 \mu\text{m}$ ($n = 6$; Fig 2B). Two weeks later in the control mice, increasing the tubular NaCl concentration from 10 to 80 mmol/L in the isolated perfused JGAs, constricted the Af-Art from 17.1 ± 0.7 to $13.3 \pm 0.6 \mu\text{m}$ (Fig 2A). TGF was $3.8 \pm 0.3 \mu\text{m}$. ($n = 6$; Fig 2B) and in the Ang II treated mice, the Af-Art constricted from 15.8 ± 0.7 to $10.6 \pm 0.9 \mu\text{m}$ (Fig 2A). TGF was $5.2 \pm 0.2 \mu\text{m}$ (* $P < 0.01$ vs saline-infusion and basal; $n = 6$; Fig 2B).

Figure 3. Representative experiments for measurement for O_2^- and NO with fluorescent dyes
 A. The macula densa plaque was loaded with dihydroethidium and oxyethidium was measured with Ex/Em at 565/605 nm. B. A light microscopy image for dihydroethidium loading. C. The macula densa plaque was loaded with DAF-2 DA and NO was measured with Ex/Em at 490/540 nm. D. A light microscopy image for DAF-2 DA loading. G: glomerulus arrow: macula densa plaque

Figure 4. O_2^- generation by the macula densa in normal and hypertensive mice

O_2^- was measured in the isolated perfused juxtaglomerular apparatus (JGA) using dihydroethidium in mice infused with Ang II or saline for 2 weeks and compared them to basal values. The macula densa was perfused with 80 mmol/L NaCl solution. O_2^- generation by the macula densa was undetectable in mice infused with saline and at basal conditions. However, O_2^- generation by the macula densa increased to 21.4 ± 2.5 unit/min in Ang II-induced hypertensive mice (* $p < 0.01$, $n = 7$).

Figure 5. O_2^- generation by the macula densa cells in the presence of NO inhibition

The previous experiments were repeated but this time in the presence of a non-selective nitric oxide synthase (NOS) inhibitor, L-NAME (10^{-4} mol/L) in tubular lumen and bath. Following perfusion of the macula densa with 80 mM NaCl solution, in basal conditions the O_2^- generation by the macula densa was 12.3 ± 1.1 unit/min. In saline infused mice, it was 14.7 ± 2.0 unit/min. In the slow-pressor Ang II infused hypertensive mice, the O_2^- generation by the macula densa increased to 45.3 ± 3.7 unit/min (* $p < 0.01$, $n = 5$).

Figure 6. NO generation by the macula densa in normal and hypertensive mice

NO was measured in the isolated perfused juxtaglomerular apparatus (JGA) using DAF-2 in mice infused with Ang II or saline for 2 weeks and compared them to basal values. The macula densa was perfused with 80 mmol/L NaCl solution. NO generation by the macula densa was 138.5 ± 9.3 and 143.9 ± 10.5 unit/min in mice at basal conditions and infused with saline. However, NO generation by the macula densa was undetectable in Ang II-induced hypertensive mice (* $p < 0.01$, $n = 7$).

Figure 7. NO generation by the macula densa cells in the presence of O_2^- scavenger

The previous experiments were repeated in separate JGA preparations, but this time in the presence of tempol (a O_2^- scavenger, 10^{-4} mol/L) in the bath and lumen. When the macula densa was perfused with 80 mM NaCl solution, the NO generation by the macula densa was 168.4 ± 12.4 unit/min under basal conditions (without infusion of Ang II or saline). In mice infused with two weeks of saline, NO generation by the macula densa was 172.6 ± 13.8 unit/min. In the Ang II infused animals, NO generation by the macula densa was 215.1 ± 14.9 unit/min (* $p < 0.01$, $n = 6$).