Enhanced expression and activity of Nox2 and Nox4 in the macula densa in ANG II-induced hypertensive mice

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Zhang J, Chandrashekar K, Lu Y, Duan Y, Qu P, Wei J, Juncos LA, Liu R. Enhanced expression and activity of Nox2 and Nox4 in the macula densa in ANG II-induced hypertensive mice. Am J Physiol Renal Physiol 306: F344–F350, 2014. First published November 27, 2013; doi:10.1152/ajprenal.00515.2013.—NAD(P)H oxidase (Nox)2 and Nox4 are the isoforms of Nox expressed in the macula densa (MD). MD-derived superoxide (O2·−) primarily generated by Nox2, is enhanced by acute ANG II stimulation. However, the effects of chronic elevations in ANG II during ANG II-induced hypertension on MD-derived O2·− are unknown. We infused a slow pressor dose of ANG II (600 ng·min−1·kg−1) for 2 wk in C57BL/6 mice and found that mean arterial pressure was elevated by 22.3 ± 3.4 mmHg (P < 0.01). We measured O2·− generation in isolated and perfused MDs and found that O2·− generation by the MD was increased from 9.4 ± 0.9 U/min in control mice to 34.7 ± 1.8 U/min in ANG II-induced hypertensive mice (P < 0.01). We stimulated MMDD1 cells, a MD-like cell line, with ANG II and found that O2·− generation increased from 921 ± 91 to 3,687 ± 183 U·min−1·106 cells−1, which was inhibited with apocynin, oxypurinol, or NS-398 by 46%, 14%, and 12%, respectively. We isolated MD cells using laser capture microdissection and measured mRNA levels of Nox2 and Nox4 and found that O2·− generation in the MD was increased 12%, respectively. We isolated MD cells using laser capture microdissection and measured mRNA levels of Nox. Nox2 and Nox4 and 12%, respectively. We isolated MD cells using laser capture microdissection and measured mRNA levels of Nox. Nox2 and Nox4.

METHODS

All procedures and experiments were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. All chemicals were purchased from Sigma (St. Louis, MO) except as otherwise indicated.
Telometry transmitter implantation. Briefly, C57BL/6 mice (18–20 g. Harlan) were anesthetized with inhaled isoflurane (Butler Chemicals). A small incision was made in the middle of the neck for the insertion of the telemetry transmitter (PA-C10, Data Sciences). 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. Mice were allowed to recover for 10 days.

Minipump insertion. Osmostic minipumps (Alzet) were primed and filled with either ANG II (600 ng·kg\(^{-1}\)·min\(^{-1}\)) or saline (control). On the day of surgery, the animals, which had 10 days to recover from the telemetry transmitter implantation, were again anesthetized with isoflurane. Under sterile conditions, a small incision was made in the midscapular 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. After insertion, the wound was sutured, and animals were allowed to recover for a day. Measurements of mean arterial pressure (MAP) were started on the second day after minipump implantation at 10-min intervals.

Laser capture microdissection and real-time PCR. Laser capture microdissection (LCM) and real-time PCR were used to isolate MD cells and to measure Nox2 and Nox4 mRNA levels with methods we have previously described (19, 37, 64). Briefly, kidneys from C57BL/6 mice were removed and snap frozen in Optimal Cutting Temperature medium. Frozen sections (8 μm thick) were obtained and then stained and dehydrated using an Arcturus Histogone frozen section staining kit (Life Technologies, Carlsbad, CA). MD cells were dissected with an Arcturus LCM System (Model Veritas). Real-time PCR was used to quantify mRNA levels of Nox2 and Nox4. Total RNA from MMDD1 cells was extracted with the RNeasy Micro kit (Qiagen) following the manufacturer’s instructions. One microgram of total RNA was reverse transcribed at 25°C for 5 min, 42°C for 45 min, and 85°C for 5 min using the iScript cDNA Synthesis kit (Bio-Rad) following the manufacturer’s instructions. Real-time PCR was performed in a C1000TM Thermal Cycler real-time PCR machine (Bio-Rad). β-Actin was used as a housekeeping gene.

Isolation and microperfusion of the JGA and measurement of \(O_2^−\) with dihydroethidium. We used methods similar to those we have previously described to isolate and microperfuse the MD with the attached glomerulus (19, 36). Briefly, the kidneys were removed and sliced along the corticomedullary axis. Single superficial intact glomeruli were microdissected together with adherent tubular segments consisting of portions of the TAL, MD, and early distal tubule. Using a micropipette, samples were transferred to a temperature-regulated chamber mounted on an inverted microscope (TE2000-S, Nikon). The glomeruli were positioned so the MD could be visualized using a holding pipette. TALs were cannulated with an array of glass pipettes.

MDs were perfused with physiological saline solution containing (in mmol/l) 10 HEPES, 1.0 CaCO\(_3\), 0.5 KHPO\(_4\), 4.0 KHCO\(_3\), 1.2 MgSO\(_4\), 5.5 glucose, 0.5 Na acetate, and 0.5 Na lactate (pH 7.4); NaCl was 10 or 80 mmol/l. The luminal perfusion rate was 40 ml/min and was controlled with a pump (11 plus, Harvard Apparatus, Holliston, MS). The bath consisted of modified essential medium containing 0.15% BSA and was exchanged continuously at a rate of 1 ml/min.

We used the \(O_2^−\)-sensitive fluorescent dye dihydroethidium to detect \(O_2^−\) levels, as we have previously described (33, 34). Briefly, once the TAL was perfused, MD cells were loaded with 10 μM dihydroethidium in 0.1% DMSO plus 0.1% pluronic acid from the lumen for 30 min and then washed for 20 min. Loaded MD cells were exposed to 380- and 490-nm light to excite dihydroethidium and oxymethidium, respectively. Emitted fluorescence from dihydroethidium was recorded using a 420-nm dichroic mirror with a 460/50-nm band-pass filter; for oxymethidium, we used a 565-nm dichroic mirror with a 605/55-nm band-pass filter. Square-shaped regions of interest were set inside the cytoplasm of MD cells, and their mean intensity was recorded every 5 s for 2 min. We recorded and calculated the rate of the changes for oxymethidium/dihydroethidium as an indicator of \(O_2^−\) production. Since we have previously found that increased luminal NaCl induced NO release by the MD (32, 34), we added the NO synthase inhibitor N-nitro-L-arginine methyl ester (10 \(^{-4}\) mol/l) to the bath and lumen while measuring \(O_2^−\) to eliminate its reaction with NO, as we have previously reported (33, 34).

Measurement of \(O_2^−\) with lucigenin-enhanced chemiluminescence in MMDD1 cells. Our experiments used MMDD1 cells, a MD-like cell line (kindly provided by Dr. J. Schnermann, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) (62), in a manner similar to our previously described studies (20, 64, 65). MMDD1 cells were stimulated by ANG II (10\(^{-6}\)–10\(^{-10}\) mol/l) for 12 h. \(O_2^−\) production in MMDD1 cells was determined using a lucigenin-enhanced chemiluminescence assay, as previously described (20, 64). Briefly, MMDD1 cells (10-cm dish) were washed with PBS twice, trypsinized in the dish, and kept in 9 or 12 ml Krebs-HEPES buffer [which contained (in mmol/l) 115 NaCl, 20 HEPES, 1.17 K-HPO\(_4\), 1.17 MgSO\(_4\), 4.3 KCl, 1.3 CaCl\(_2\), 25 NaHCO\(_3\), 11.7 glucose, and 0.1 NAD(P)H, with pH adjusted to 7.4]. Lucigenin (5 \(\times\) 10\(^{-5}\) mol/l) was then added to the samples, which were incubated for 30 min at 37°C with oxygen bubbling. A 0.5-ml sample was transferred into 1.6-ml polypropylene 8-tube, and, using a Sirius luminescence (Berthold Detection Systems, Pforzheim, Germany), \(O_2^−\) was then measured following the manufacturer’s instructions. Luminescence was measured for 10 s with a delay of 5 s.

Preparations for small interfering RNA. Nox2, Nox4, and p22\(^{phox}\) small interfering (si)RNAs were designed and synthesized by Santa Cruz Biotechnology (Santa Cruz, CA). siRNA transfection was performed following the manufacturer’s instructions as previously described (64, 65). Briefly, the transfection of the siRNA was performed using TransMessenger Transfection Reagent from QIANGEN (Germantown, MD) according to the manufacturer’s instructions. Scrambled siRNAs were synthesized and used as negative controls. Twenty-four hours before transfection, MMDD1 cells were transfected onto six-well plates and transfected with 2 μg of each siRNA duplex using TransMessenger Transfection Reagent for 3 h in medium devoid of serum and antibiotics. MMDD1 cells were then washed once with PBS and grown in complete medium. Gene silencing was monitored by measuring RNA after incubation for 24 h.

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’s least-significant-difference test or a Student’s paired t-test when appropriate. Changes were considered to be significant if \(P < 0.05\). Data are presented as means ± SE.

RESULTS

Suppressor ANG II infusion induced hypertension in mice. To demonstrate the effect of a slow pressor dose of ANG II on blood pressure, we infused ANG II with a minipump at a dose of 600 ng·min\(^{-1}\)·kg\(^{-1}\) for 2 wk and measured MAP with telemetry in C57BL/6 mice. We found that basal MAP was 101 ± 3.1 mmHg and that this started to gradually elevate from day 5 of minipump insertion in the ANG II-treated group. Blood pressure continued to rise and reached a peak of 123 ± 3.8 mmHg on days 10–14 of the infusion (\(n = 7\), \(P < 0.01\)). MAP of control mice infused with saline did not vary significantly (\(n = 5\); Fig. 1).

\(O_2^−\) generation by the MD is enhanced in ANG II-induced hypertensive mice. To determine whether \(O_2^−\) generation by MD cells increases after ANG II infusion, we isolated and perfused the JGA and measured \(O_2^−\) generation using dihydroethidium in mice infused with ANG II or saline for 2 wk by minipumps. The MD was perfused with 80 mM NaCl solution and...
in the presence of N-nitro-L-arginine methyl ester (10⁻⁴ mol/l) in the bath and lumen. O₂⁻ generation by the MD was 9.4 ± 0.9 U/min in control mice. It increased to 34.7 ± 1.8 U/min in ANG II-induced hypertensive mice (n = 6, P < 0.01; Fig. 2).

To determine the source of O₂⁻ generation, we used the Nox inhibitor apocynin and repeated the above protocol. In the presence of apocynin, O₂⁻ generation by the MD was 2.1 ± 0.3 U/min in control mice and increased to 12.4 ± 2.3 U/min in ANG II-induced hypertensive mice (n = 5, P < 0.01; Fig. 2). These data indicate that there is a significant increase in MD O₂⁻ generation in ANG II-hypertensive mice and that Nox is an important source of this O₂⁻.

ANG II increased O₂⁻ generation in cultured MMDD1 cells.

To further study the source of ANG II-induced O₂⁻ generation in the MD, we stimulated cultured MMDD1 cells with ANG II in the presence of apocynin and repeated the above protocol. In the presence of apocynin, O₂⁻ generation by the MD was 2.1 ± 0.3 U/min in control mice and increased to 12.4 ± 2.3 U/min in ANG II-induced hypertensive mice (n = 5, P < 0.01; Fig. 2). These data indicate that there is a significant increase in MD O₂⁻ generation in ANG II-hypertensive mice and that Nox is an important source of this O₂⁻.

Fig. 2. Superoxide (O₂⁻) generation by the macula densa (MD) is enhanced in ANG II-induced hypertensive mice. In the isolated and perfused juxtaglomerular apparatus, O₂⁻ generation by the MD was 9.4 ± 0.9 U/min in mice infused with saline. It increased to 34.7 ± 1.8 U/min in mice infused with ANG II for 2 wk via minipumps (n = 6). In the presence of apocynin, a NAD(P)H oxidase (Nox) inhibitor, O₂⁻ generation by the MD was 2.1 ± 0.3 U/min in control mice and increased to 12.4 ± 2.3 U/min in ANG II-induced hypertensive mice (n = 5). #P < 0.01 and *P < 0.05 vs. without apocynin.

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levels were measured with real-time PCR. As shown in Fig. 5, Nox2 mRNA levels in the MD increased 3.7 ± 0.17-fold in ANG II-infused mice compared with control mice (n = 5, P < 0.01). Nox4 mRNA levels in the MD increased 2.6 ± 0.15-fold in the ANG II-infused mice compared with control mice (n = 5, P < 0.01; Fig. 5). These data indicate that both Nox2 and Nox4 isoforms of Nox in the MD significantly increased in subpressor ANG II-induced hypertension.

ANG II-generated \( \text{O}_2^- \) in the MD originates from both Nox2 and Nox4 isoforms of Nox in MMDD1 cells. To determine which isoform(s) of Nox is the primary source of ANG II-stimulated \( \text{O}_2^- \) generation, we used Nox2 and Nox4 siRNAs to silence Nox2 and Nox4 genes and used p22\(^{phox}\) siRNA to knock down the function of both Nox2 and Nox4. ANG II stimulation increased \( \text{O}_2^- \) generation in MMDD1 cells treated with scrambled siRNA from 967 ± 42 to 3,278 ± 94 U-min\(^{-1}\)10\(^5\) cells\(^{-1}\). In MMDD1 cells treated with Nox2 or Nox4 siRNAs, ANG II-stimulated \( \text{O}_2^- \) generation was 1,632 ± 61 and 1,932 ± 57 U-min\(^{-1}\)10\(^5\) cells\(^{-1}\), respectively. In cells treated with p22\(^{phox}\) siRNA, ANG II-stimulated \( \text{O}_2^- \) generation was blocked (784 ± 83 U-min\(^{-1}\)10\(^5\) cells\(^{-1}\); Fig. 6A). To further confirm the contributions from Nox2 and Nox4, we treated MMDD1 cells with both Nox2 and Nox4 siRNAs. ANG II stimulation increased \( \text{O}_2^- \) generation in MMDD1 cells treated with scrambled siRNAs from 853 ± 51 to 2,841 ± 83 U-min\(^{-1}\)10\(^5\) cells\(^{-1}\). In MMDD1 cells treated with Nox2 and Nox4 siRNAs, ANG II-stimulated \( \text{O}_2^- \) generation was 718 ± 44 U-min\(^{-1}\)10\(^5\) cells\(^{-1}\) (Fig. 6B). These data indicate that ANG II enhances \( \text{O}_2^- \) generation from both Nox2 and Nox4 isoforms of Nox in MMDD1 cells.

**DISCUSSION**

ANG II, the key effector peptide of the renin-angiotensin system, is vital in the initiation and progression of hypertension, vascular hypertrophy, and atherosclerosis (29). These actions of ANG II, including vascular smooth muscle constriction, elevated systemic blood pressure, endothelial dysfunction, vascular remodeling, and retention of Na\(^+\), are mediated through ROS generated by Nox (23, 63). ROS are generated after ANG II-induced activation of ANG II type 1 (AT\(_1\)) receptors. Indeed, most effects of ANG II in the MD (enhancing TGF and increasing intracellular Ca\(^{2+}\)) are mediated through AT\(_1\) receptors. ANG II influences the O\(_2\) anion, an important signaling element for hypertension and other deleterious actions of ANG II. We previously used acute doses of ANG II to demonstrate that ANG II increases O\(_2\) in the MD and that the source of this O\(_2\) is Nox2. These increases in O\(_2\) enhanced TGF by quenching NO at the MD (20, 64). This raised the possibility that in situations associated with increased ANG II sensitivity and abnormal Na\(^+\) handling may be mediated by Rac-enhanced increases in O\(_2\). However, these studies also used acute ANG II, which does not mimic pathophysiological conditions where ANG II is chronically elevated. In this study, we accomplished this by chronically infusing subpressor doses of ANG II. This chronic ANG II induces oxidative stress and salt-sensitive hypertension, increases pro-inflammatory factors, and leads to progressive renal injury (11–14, 16, 18, 24, 25, 31, 43, 44, 48, 49). Indeed, this chronic ANG II model is recognized as 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 (8, 17, 22, 52). The dose needed to cause these subpressor ANG II-induced changes varies based on the route and species of animals. In mice, it fluctuates anywhere from 400 to 1,000 ng·kg\(^{-1}\)min\(^{-1}\) (2, 5, 9, 10, 15, 52, 57). In our study, we used ANG II at a dose of 600 ng·min\(^{-1}\)·kg\(^{-1}\) since we found that this dose consistently elevated MAP by about 20 mmHg (Fig. 1). While this dose elevated blood pressure, it is not as well known whether subpressor ANG II alters oxidative stress at the MD level and, if so, what the primary sources are for ANG II-induced O\(_2\) at the MD. Hence, the aim of this study was to determine the changes in the expression as well as function of Nox2 and Nox4 in the MD after chronic ANG II stimulation. The key findings from our present study are that both Nox2 and...
From chronic ANG II-treated mice, we noted that chronic ANG II-induced O$_2^-$ generation in MMDD1 cells treated with scrambled siRNAs from 853 to 3,278 units/min/10$^5$ cells treated with scrambled small interfering (si)RNA from 1,632 to 57 units/min/10$^5$ cells treated with scrambled siRNA from 967 to 23). In these cells, ANG II elevated O$_2^-$ levels in human embryonic kidney-293 cells transfected with Nox2 and p47. These data clearly show that apocynin is not simply an O$_2^-$ scavenger. In our preparation, we used apocynin at a concentration of 10$\mu$M. Given that this concentration is 100 times less than that needed to scavenge O$_2^-$, it is unlikely that apocynin acts via this mechanism in our preparation. We also realize that the concentration of oxypurinol we used in this study was pretty high, which could act as an antioxidant (3).

Our previous studies have demonstrated that the two isoforms of Nox in the MD are Nox2 and Nox4. To confirm this, we used LCM techniques to isolate the MD, as previously described (64). Real-time PCR was used to measure Nox2 and Nox4 mRNA levels. We noted that ANG II-treated mice had an about fourfold increase in Nox2 and a threefold increase in Nox4 compared with control mice. This suggests that both Nox2 and Nox4 isoforms of Nox are increased in chronic ANG II-induced hypertensive mice. Next, to isolate which isoform(s) of Nox is involved in mediating the chronic ANG II-induced O$_2^-$ generation; this suggests that unlike acute ANG II stimulation, chronic ANG II stimulates both Nox2 and Nox4 in the MD to generate O$_2^-$.

We noted that subpressor ANG II-induced O$_2^-$ generation was inhibited in MMDD1 cells treated with scrambled siRNAs from 853 to 57 units/min/10$^5$ cells treated with scrambled siRNA from 967 to 23). In these cells, ANG II stimulation increased O$_2^-$ generation; this suggests that unlike acute ANG II stimulation, chronic ANG II stimulates both Nox2 and Nox4 in the MD to generate O$_2^-$.

Moreover, O$_2^-$ from these cells was completely blocked, signifying that both Nox2 and Nox4 are activated by ANG II. Compared with Nox2, there is a paucity of studies defining the significance and mechanism of action of the Nox4 isoform. A study (38) on Nox4 has suggested that it is the primary source of O$_2^-$ in TALs after acute ANG II stimulation. Welch et al. (41) infused rats with chronic ANG II, measured SNGFR in rats treated with siRNA against p22phox and found that SNGFR in these animals was significantly lowered, implying that Nox plays a very significant role in the regulation of TGF-mediated SNGFR. Moreover, unlike Nox2, Nox4 is also a significant source of H$_2$O$_2$, which is essential in the regulation of vascular activity (1, 47, 55).

In the present study, we mainly focused only on the generation of O$_2^-$. Limitations of the present study are that we were unable to...
perform all the protocols in vivo and in isolated perfused JGAs. We had to use a cell line, namely, MMDD1 cells. We expect that MMDD1 cells will yield results that are complementary with the results obtained in perfused JGAs and in vivo. We realize that, phenotypically, the MMDD1 cell line may not be exactly the same as actual MD cells in vivo (26). Any end-point differences between MMDD1 and actual MD cells should be interpreted with caution.

Although salt sensitivity is considered to be primarily an isolated defect in pressure natriuresis, along with this deficiency there exists an important physiological mechanism that plays a part in the renal physiological and structural changes leading to salt sensitivity. This mechanism is known as resetting the set point of TGF. In humans, resetting TGF contributes to the development of salt-sensitive hypertension (4, 30, 46). In these hypertensive models, administration of angiotensin-converting enzyme inhibitors or AT1 receptor blockers significantly reduce the sensitivity of the TGF mechanism (6, 28, 39, 45), and peritubular infusions of ANG II enhanced the TGF generation in the MD. The source of this $O_2^-$ is primarily from Nox. Moreover, our data also indicate that chronic ANG II stimulates both Nox2 and Nox4.

REFERENCES