Angiotensin II-induced hypertension blunts thick ascending limb NO production by reducing NO synthase 3 expression and enhancing threonine 495 phosphorylation

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ANGIOTENSIN II (ANG II) affects many systems, including the cardiovascular, renal, and central nervous systems. In experimental models, doses of ANG II that have no measurable effects on blood pressure, urinary volume, and urinary Na excretion acutely have profound effects on these parameters when infused chronically (14, 27, 49, 64). Such models of ANG II-induced hypertension have been widely used to study both the causes (1, 2, 12, 17, 27, 44) and consequences (9, 14, 28, 30) of hypertension.

A large range of doses has been used to induce ANG II-dependent hypertension in rats. Generally, lower doses of ANG II (5–200 ng·kg−1·min−1) produce a salt-sensitive form of hypertension (75), a disease in which dietary salt increases blood pressure, whereas higher doses of ANG II (>400 ng·mg−1·min−1) produce immediate increases in blood pressure that no longer depend on salt intake (75). In salt-sensitive forms of hypertension induced by ANG II, animals are in positive Na balance from 3 to 10 days (25, 75). The Na retention may be at least in part due to reductions in the effects of nitric oxide (NO) in the kidney, since the prohypertensive actions of ANG II are specifically mitigated by NO (5, 12, 54, 76). In addition, decreased NO production and signaling in the renal medulla are associated with salt-sensitive hypertension (18, 26, 38, 50, 51, 65, 80).

The thick ascending limb of the loop of Henle reabsorbs ~25% of the filtered NaCl load, and NO acts as an autacoid regulating this process (23, 57, 58, 61, 68–70, 83). All isoforms of NO synthases (NOS1, 2, and 3) are expressed in thick ascending limbs. Stimulation of NO production with the general NOS inhibitor L-NAME decreases transport in wild-type mice (69). This effect is preserved in NOS1 or NOS2 knockout mice, but is blunted in NOS3 knockout mice (62, 69). These data indicate that NO from NOS3 regulates transport in this nephron segment (13, 62, 66, 69). NOS3 activity can be regulated by changes in expression and phosphorylation in the thick ascending limb (35, 73). We showed that ANG II reduces (72) while a high-salt diet initially increases (33, 35), and then diminishes NOS3 expression (35). Several factors acutely alter phosphorylation including endothelin-1 (34, 71), ATP (6, 78), and luminal flow (6, 59, 60). These factors stimulate thick ascending limb NO production via phosphatidylinositol 3,4,5-tri-phosphate (PIP3) and phosphorylation of NOS3 at serine 1177 (S1177). In contrast, phosphorylation at threonine 495 (T495) and serine 1177 (S1177), inhibitory and stimulatory sites, respectively. We hypothesized that NO production in the thick ascending limbs is impaired by chronic ANG II infusion, due to reduced NOS3 expression, and phosphorylation at threonine 495. The decreased NO production observed in ANG II-infused rats is blunted in NOS3 knockout mice (62, 69). These findings are consistent with the idea that NO from NOS3 regulates transport in this nephron segment (13, 62, 66, 69).

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We hypothesized that NO production in the thick ascending limb is impaired by chronic ANG II infusion, due to reduced NOS3 expression, increased phosphorylation of T495, and decreased phosphorylation at S1177.

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METHODS

Drugs and buffers. Unless specified, all drugs and reagents were obtained from Sigma (St. Louis, MO). The NO-sensitive dye 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate) was obtained from Invitrogen (Grand Island, NY). Cosmogen Plus Protein Assay Reagent was obtained from Thermo-Scientific (Rockford, IL). ANG II and endothelin-1 were obtained from Bachem (Torrance, CA). Phosphatidylinositol 3,4,5-triphosphate (PIP₃) and neomycin sulfate (carrier) were obtained from Echelon Biosciences (Salt Lake City, UT).

HEPES-buffered physiological saline (physiological saline) used for kidney perfusion and to generate thick ascending limb suspensions contained (in mmol/l) 10 HEPES (pH 7.5), 130 NaCl, 4 KCl, 2.5 Na₂HPO₄, 1.2 MgSO₄, 5.5 glucose, 6.0 DL-alanine, 2.0 Ca(lactate)₂, and 1.0 Na₃citrate. Osmolality was adjusted to 290 ± 5 mosmol/kgH₂O.

Lysis buffer contained 20 mmol/l HEPES (pH 7.5), 2 mmol/l EDTA, 300 mmol/l sucrose, 1.0% Igepal, 0.1% Na dodecyl sulfate, 5 mg/l antipain, 5 mg/l aprotinin, 5 mg/l leupeptin, 5 mg/l chymostatin, 5 mg/l pepstatin A, 4 mmol/l benzamidine, and 116 mmol/l pF-block (Sigma). Milk blocking buffer contained 50 g/l nonfat milk dissolved in 20 mmol/l Tris (pH 7.6), 137 mmol/l NaCl, and 0.1% Tween-20. The blocking buffer Blok-PO, the phosphatase inhibitor Cocktail Set II, and the Luminata luminol-based chemiluminescent substrate for horseradish peroxidase were obtained from EDM Millipore (San Diego, CA).

Animal model. This study was approved by the Institutional Animal Care and Use Committee of the Henry Ford Hospital, Wayne State University, and Case Western Reserve University. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 90–120 g were infused with either vehicle (0.01 mol/l acetic acid) or ANG II (200 ng·kg⁻¹·min⁻¹). After 1 or 5 days, rats of this size were used so that thick ascending limbs could be dissected after ANG II infusion. Minipump implantation was performed under isofluorane anesthesia (1.5% in O₂, flow 0.5 l/min). At day 5, animals were anesthetized with ketamine (100 mg/kg body wt ip) and xylazine (20 mg/kg body wt ip). Animals used to prepare thick ascending limb suspensions were also given 2 IU heparin ip. Mean arterial blood pressure (MAP) was measured under anesthesia as we reported before (24).

Thick ascending limb suspension preparation. Thick ascending limb suspensions were generated as we described (24, 73). Briefly, the abdominal cavity was opened and the kidneys were flushed with cold physiological saline containing 2.5 U/ml of heparin and 0.1% type I collagenase were excised and cut in coronal slices. The inner stripe of the outer medulla was recovered by centrifugation (100 g, 2 min, 4°C) and the pellet was resuspended in fresh physiological saline and stirred on ice for 30 min. After being stirred, the tubules were filtered through a 250-μm nylon mesh, recovered from the filtrate by centrifugation (100 g, 2 min, 4°C) and rinsed two times with cold physiological saline. The resulting product yields ~95% pure suspension of thick ascending limbs (10, 32).

Western blot. Western blots were conducted using similar techniques as before (20, 24, 72). Unless otherwise noted, samples were processed as follows: thick ascending limb suspensions were dissolved in lysis buffer and the debris was removed by centrifugation (5,600 g, 5 min, 4°C). Protein was determined by a colorimetric assay. Polyacrylamide gels (6%) were used. Equal amounts of protein from freshly prepared lysates were loaded per lane. Samples from vehicle and ANG II-infused rats were processed in pairs and loaded in the same gel, so each gel had its own control. For ET₁ receptor samples, a 4% stacking/9% running gel combination was used. For T495 phosphorylation, paired samples were loaded in nonadjacent lanes of the same gel. Optical density of each band was expressed as a fraction of the total density. Values were corrected for loading using the same procedure as depicted below. Western blots were conducted as specified in Table 1. For loading control, the membranes were stripped with 200 mmol/l glycine (pH 2.8) for 30 min and rebotted for β-tubulin in the case of NOS3 and ET₁ receptor, and NOS3 in the case of phospho-NOS3 (S1177) and (T495).

Protein detection and densitometry. The signal was detected by exposing a Fuji Super RX film to the membranes. Films were scanned using an EPSON Expression 1680 scanner with EPSON-Scan software (positive film, 16-bit grayscale, 600 dpi). Densitometry of the bands was measured using a custom-made software. The exposure times for the films were standardized so that the mean optical density of the bands was between 0.40 and 1.00.

Measurement of NO production. NO production was measured by fluorescence microscopy as we have done before (7, 8, 31, 60, 62). In brief, thick ascending limbs were manually dissected, transferred to a temperature-controlled chamber (37 ± 1°C), and held by two glass pipettes without luminal flow. The bath contained 100 mmol/l L-arginine. Under these conditions, basal NO production by the tubules is negligible (59, 60). Tubules were loaded with DAF-FM (4 μmol/l) for 20 min and then rinsed for 20 min. For imaging, a ×40 oil immersion objective was used. The dye was excited by a xenon arc lamp with a 488-nm band pass filter. The fluorescence emitted by the NO-bound dye (>500 nm) was measured using Metafluor software (Molecular

Table 1. Antibodies and blotting conditions

| Antibody      | Provider      | Catalog Number | Source       | Blocking Buffer | Dilution | Buffer | Time | Temp.
|---------------|---------------|----------------|--------------|----------------|----------|--------|------|------|
| NOS3          | BD Biosciences| 610296         | Mouse        | 5% Milk†       | 1:1,000   | 5% Milk| 1 h  | Room T.
| pNOS3 (S1177) | BD Biosciences| 612392         | Mouse        | 5% Milk       | 1:2,000   | 5% Milk| 1 h  | Room T.
| pNOS3 (T495)  | CellSignaling | 9574           | Rabbit       | 5% Milk       | 1:500     | BlokTM-PO*| 15 h | 4°C  |
| ET₁ receptor  | Alomone       | AER-002        | Rabbit       | 5% Milk       | 1:2,500   | 5% Milk| 1 h  | Room T.
| β-Tubulin     | Abcam         | ab6046         | Rabbit       | 5% Milk       | 1:10,000  | 5% Milk| 1 h  | Room T.
| 2' anti-rabbit-HRP | GE Healthcare  | NA9340V       | Donkey       | 5% Milk       | 1:2,500   | 5% Milk| 1 h  | Room T.
| 2' anti-mice-HRP | GE Healthcare  | NA931V        | Sheep        | 1:1,000       | 5% Milk   | 1 h    | Room T.

*Blok TM-PO (Millipore, cat. no.: WBAVDP001). †5% Milk: 50 g/l nonfat milk dissolved in 20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween 20.
distribution. Measurements were recorded once every 30 s for a 10-min control period. Then treatments were applied, and fluorescence was measured under the same conditions during a 10-min experimental period. Given that dye binding to NO is irreversible, the change in fluorescence with time represents NO production. A linear regression of the 5 min of maximal stimulation during the experimental period was performed and compared with the last 5 min of the control period. The difference was expressed as arbitrary fluorescence units (AFU)/min and represents the NO response to the treatment. Treatments were performed and compared with the last 5 min of the control period.

Data analysis. Results are expressed as means ± SE. Paired t-test and 2-sample Wilcoxon tests were used as appropriate. When multiple pair-wise comparisons were made, Hochberg’s method for multiple test of significance was used. P < 0.05 was considered significant.

RESULTS

Rats infused with 200 ng·kg⁻¹·min⁻¹ of ANG II for 5 days had increased MAP compared with vehicle-infused rats (Vehicle: 96 ± 4 mmHg, n = 5 vs. ANG II: 120 ± 8 mmHg in ANG II, n = 4; P < 0.04). One-day infusion of ANG II did not produce any measurable change in MAP (Table 2).

We previously showed that in vitro ANG II treatment decreases NOS3 expression in primary cultures of thick ascending limbs (72); therefore, we next measured NOS3 expression in thick ascending limbs from ANG II-infused and control animals. We found that infusion of ANG II for 5 days decreased NOS3 expression by 40 ± 12% (P < 0.007; n = 6; Fig. 1) compared with controls infused with vehicle. ANG II had no significant effect when infused for 1 day (Δ = −7 ± 18%; n = 5).

Several studies have shown that PKC mediates at least some of the effects of ANG II in thick ascending limbs (3, 36, 79, 85). Since PKC targets NOS3 at the inhibitory site T495 (37, 48), we next measured NOS3 phosphorylation at this residue. We found that 5-day infusion of ANG II increased NOS3 phosphorylation at T495 by 147 ± 26% (P < 0.008; n = 6) compared with the controls infused with vehicle (Fig. 2). In contrast, T495 phosphorylation did not change in thick ascending limbs from rats infused with ANG II for 1 day (Δ = 13 ± 20%; n = 5).

Given that ANG II infusion altered NOS3 expression and phosphorylation only after 5 days of infusion, we studied whether these effects reduce NO production in response to physiological stimuli at this time point. We tested whether the ability of 1 nmol/l endothelin-1 to stimulate NO production was impaired in thick ascending limbs from ANG II hypertensive rats. Endothelin-1 increased NO production in thick ascending limbs from vehicle-infused rats by 0.17 ± 0.02 AFU/min (n = 8). In contrast, endothelin-1 did not significantly affect NO production in tubules from ANG II-infused animals (−0.01 ± 0.06 AFU/min; n = 7; vehicle vs. ANG II: P < 0.01; Fig. 3).

Endothelin-1 increases NO by binding ETB receptors. Therefore, the diminished response to endothelin-1 could be a result of a reduction in ETB receptors. We found that chronic ANG II infusion did not change thick ascending limb ETB receptor expression compared with controls (Δ = 10 ± 10%; n = 4; Fig. 4).

PIP3 mediates the stimulatory action of many factors that activate NOS3 in thick ascending limbs and use of PIP3 as a NOS3 activator bypasses many of the signaling steps that could be altered by ANG II-induced hypertension. Thus, we next tested whether PIP3-induced NO production is reduced by ANG II infusion. In thick ascending limbs from vehicle-treated animals PIP3 increased NO production by 0.13 ± 0.04 AFU/min (n = 4); however, it had no significant effect on NO production in tubules from ANG II-infused animals (Δ =
Fig. 3. Endothelin-1-induced NO production as measured by DAF-FM fluorescence in isolated thick ascending limbs obtained from ANG II- and vehicle-infused rats after 5 days of treatment. The NO response to endothelin-1 stimulation was blunted in thick ascending limbs from rat infused ANG II (−0.01 ± 0.06; n = 7) compared with controls (0.17 ± 0.06; n = 8, P < 0.01).

−0.07 ± 0.06 AFU/min, n = 4; Control vs. ANG II: P < 0.03; Fig. 3).

The final step in NOS3 activation by PIP3 is phosphorylation at S1177. Thus, we tested whether PIP3-induced phosphorylation of NOS3 at S1177 was reduced in ANG II-induced hypertension. In vehicle-treated rats, PIP3 augmented phosphorylation by 18 ± 4% (P < 0.02; n = 5; Fig. 6A). In contrast, PIP3 had no significant effect on thick ascending limbs from ANG II-treated animals (Δ = −1 ± 18%; Fig. 6B).

DISCUSSION

NO production by NOS3 in thick ascending limbs is a critical factor in blood pressure and salt handling by the kidney. Since NO antagonizes the actions of ANG II in the kidney, we hypothesized that NO production by thick ascending limbs is impaired by chronic ANG II infusion due to downregulation in NOS3 expression and altered phosphorylation.

First, we measured NOS3 expression in medullary thick ascending limb suspensions by Western blot. We found that chronic ANG II infusion decreased NOS3 expression by 40% compared with vehicle at day 5, but had no effect at day 1. This raises the question of whether the decrease in NO is a result of ANG II accumulation or the associated increase in blood pressure. Some insights to answer this question come from the 2 kidney-1 clip hypertension model, where each kidney is exposed to similar ANG II levels (84) but different perfusion pressures. In this model, NOS3 decreased to the same extent in the medulla of the clipped and contralateral kidneys (86). These data indicate that some factor other than blood pressure reduces NOS3 expression, likely the elevated ANG II. In addition, ANG II decreased NOS3 expression in primary cultures of thick ascending limbs (72) where blood pressure, shear stress, and hypoxia are absent. Taken together, these data suggest that chronic elevations in ANG II rather than the associated increase in blood pressure are responsible for decreasing NOS3 expression in the thick ascending limb.

Our data showing that ANG II decreases NOS3 expression at 5 days contrast with those of others showing that ANG II infusion did not change (11, 54, 86) or increased (53) NOS3 in the renal medulla. They are also different from data showing that chronic ANG II infusions increase NOS3 expression in the renal cortex (11, 53, 54). Some insights into the variability of the results come from analyzing the effects on ANG II on other tissues. On one hand, ANG II increased NOS3 expression in the rat aorta (52) and primary cultures of bovine pulmonary artery endothelial cells (56), but not in bovine coronary artery endothelial cells (56). On the other hand, there are reports showing the opposite, ANG II decreases NOS3 expression in human coronary artery endothelial cells (45) and ANG II receptor blockade increases NOS3 expression in rat aortic endothelial cells (87). Disparate results have been also reported in the heart (42, 53, 81). Taken together, these results seem to indicate that the effects of ANG II are dose- and time-dependent.

Directly to this point, McDonough and colleagues (55) recently showed that even though ANG II acutely stimulates Na transport all along the nephron, 2-wk infusion of pressor doses of ANG II results in segment-specific effects. These investigators postulated that initially ANG II infusion produces a direct increase in salt reabsorption in all nephron segments. However, with time the increase in blood pressure reduces proximal nephron reabsorption via a pressure-natriuretic mechanism while distal nephron Na reabsorption remains elevated. In support of this, 2-wk infusion of pressor doses of ANG II...
results in elevations in cortical NO (11, 12) concomitantly with the pressure natriuretic response. Thus, these data suggest that increased NO is a compensatory response to the elevation of blood pressure, not a direct effect of ANG II.

Given that NOS3 is regulated by both expression and phosphorylation, we next studied whether phosphorylation of NOS3 at the inhibitory site T495 was affected by ANG II infusion. We found that phosphorylation of T495 increased by 147% in thick ascending limbs from animals infused with ANG II for 5 days but did not significantly change after 1 day. These data suggest that ANG II accumulation is necessary for the increase in T495 phosphorylation. In this regard, our lab showed that acute and chronic activation of angiotensin receptor 1 (AT1) increases superoxide production via PKC-α (36, 79). Since PKC-α phosphorylates NOS3 at T495 (39, 77), it is possible that the increase in T495 phosphorylation observed in our study is a result of ANG II-induced PKC activation rather than increased blood pressure.

Both lines of evidence, reduced NOS3 expression and increased phosphorylation at the inhibitory site T495, indicate that NO production by the thick ascending limbs is impaired in ANG II-induced hypertension. To test this possibility, we measured the capacity of a physiological stimulus to increase NO production by isolated thick ascending limbs. Endothelin-1 increased NO production in control tubules but not in thick ascending limbs from ANG II-treated rats.

Since endothelin-1 stimulates NO production by binding ETB receptors in the thick ascending limb (68), it is possible that the reduced response to endothelin-1 in ANG II-infused rats was due to downregulation of the receptor. Therefore, we tested whether ETB receptor expression was affected by ANG II infusion. We were unable to find any differences in ETB receptor expression among groups. Thus, reduced receptor expression cannot explain the lack of response to endothelin-1.

ATP (6, 78), α2 adrenergic agonists (67), endothelin (34, 71), and luminal flow (6, 59, 60) stimulate NO production by NOS3 in thick ascending limbs via activation of phosphatidyl inositol 3 kinase and PIP3. Since many factors that activate NOS3 in thick ascending limbs do so by elevating PIP3 (21), and use of PIP3 would avoid further confounding issues of receptor number, etc., we next examined the ability of PIP3 to stimulate NO production. We found that the increase in NO production present in control animals was absent in animals infused with ANG II. These data indicate that ANG II infusion impairs the capacity of NOS3 to respond to any physiological stimulus that acts via PIP3.

The final step in NOS3 activation by PIP3 is phosphorylation of the stimulatory site S1177. There is growing evidence of regulation of NOS3 activity by reciprocal phosphorylation of T495 and S1177 upon application of several stimuli (29, 47, 77, 82), including blockade of the AT1 receptor (4, 74). In addition, changes in NOS3 phosphorylation at S1177 have been shown in several diseases including hypertension (16, 40, 41, 43, 46). Thus, we next tested whether phosphorylation at S1177 was also affected by ANG II infusion.

Our results indicate that PIP3-induced phosphorylation of NOS3 at S1177 was impaired in thick ascending limbs from ANG II-induced hypertensive animals. Consistent with these findings in the thick ascending limb, phosphorylation of NOS3 at S1177 was found to be decreased in the aorta (87) and cerebral cortex (63) of spontaneously hypertensive rats, whereas this was restored by AT1 blockade (63, 87), indicating that ANG II signaling is involved in this process.

The data presented here indicate that changes in phosphorylation of NOS3, and thus activity, caused by ANG II are time-dependent. This hypothesis is consistent with previously published reports. We and others have shown that acute in vitro ANG II treatment stimulates NO in the thick ascending limb (15, 31). Intravenous infusion of 200 ng·kg⁻¹·min⁻¹ for 3 h to rats also produced an increase on medullary NO; however, NO production returned to values not different from controls at day 3 of infusion (53). Building on these results, our current study shows that NO production and phosphorylation of T495 are affected by day 5 of infusion of slow-pressor doses of ANG II.

In the thick ascending limb, NOS3/NO/cGMP pathway regulates salt reabsorption by retrieving NKCC2 from the plasma membrane, thereby reducing its activity. In addition, we previously reported that net transport is increased by the thick ascending limbs in ANG II-induced hypertension (79) and that the increase in thick ascending limb’s Na-K-ATPase in this model depends on a chronically increased NKCC2-dependent Na reabsorption (24). We now report that NO production is impaired in the thick ascending limbs during ANG II-induced hypertension. We speculate that defects in NO
signaling are responsible for the chronic increase in transport by the thick ascending limbs in this model. In summary, our data indicate that in ANG II-induced hypertension NO production is impaired in the thick ascending limbs, due to decreased NOS3 expression and altered phosphorylation. Lack of NO in the renal medulla contributes to the development and maintenance of salt-sensitive hypertension (22, 26, 35, 38, 50, 65), therefore, reduced NO production by this segment might be a contributing factor to increased blood pressure in this model of hypertension.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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