Angiotensin II Decreases Nitric Oxide Synthase 3 Expression via Nitric Oxide and Superoxide in the Thick Ascending Limb

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Abstract—NO produced by NO synthase type 3 (NOS3) in medullary thick ascending limbs (mTHALs) inhibits Cl− reabsorption. Acutely, angiotensin II stimulates thick ascending limb NO production. In endothelial cells, NO inhibits NOS3 expression. Therefore, we hypothesized that angiotensin II decreases NOS3 expression via NO in mTHALs. After 24 hours, 10 and 100 nmol/L of angiotensin II decreased NOS3 expression by 23±9% (n=6; P<0.05) and 50±5% (n=7; P<0.001), respectively, in primary cultures of rat mTHALs. NO synthase inhibition by 4 mmol/L of N(G)-nitro-L-arginine methyl ester hydrochloride prevented angiotensin II from decreasing NOS3 expression (Δ=−5±8%; n=5). In the presence of N(G)-nitro-L-arginine methyl ester hydrochloride, the addition of exogenous NO (1 μmol/L spermine NONOate) restored the angiotensin II–induced decreases in NOS3 expression (−22±6%; n=7; P<0.013). In addition, NO scavenging with 10 μmol/L of carboxy-PTIO abolished the effect of angiotensin II in NOS3 expression (Δ=−1±8% versus carboxy-PTIO alone; n=6). Angiotensin II increases superoxide, and superoxide scavenges NO. Thus, we tested whether scavenging superoxide enhances the angiotensin II–induced reduction in NOS3 expression. Surprisingly, treatment with 100 μmol/L of Tempol, a superoxide dismutase mimetic, blocked the angiotensin II–induced decrease in NOS3 expression (Δ=−3±7%; n=6). This effect was not because of increased hydrogen peroxide. We concluded that angiotensin II–induced decreases in NOS3 expression in mTHALs require both NO and superoxide. Decreased NOS3 expression by angiotensin II in mTHALs could contribute to increased salt retention observed in angiotensin II–induced hypertension. (Hypertension. 2009;53[part 2]:313-318.)

Key Words: reactive oxygen species  ■  oxidative stress  ■  hypertension  ■  peroxynitrite  ■  endothelial NO synthase

Thick ascending limbs (THALs) reabsorb 20% to 30% of the filtered NaCl load.1 The importance of THALs in salt-volume regulation is evidenced by the efficiency of loop diuretics, which inhibit transport in this segment, to induce natriuresis.2 NO produced by NO synthase (NOS) type 3 (NOS3 or endothelial NOS) acts as an autacoid to inhibit transport in this segment.3–5 NOS3 activity is regulated by both changes in expression6 and allosteric modulation.7,8 Thus, studying the factors that regulate NOS3 expression in THALs is of physiological relevance.

Angiotensin II (Ang II) regulates NOS3 expression in the kidney. Chronic Ang II infusion increases NO-dependent renal blood flow in the cortex,9 as well as NOS3 expression.10–12 Conversely, in the medulla, Ang II decreases NO-dependent renal blood flow,9 but the effect on NOS3 expression is controversial. In 2-kidney, 1-clip hypertension, a model of elevated Ang II, NOS3 expression is reduced in the outer medulla.11 In contrast, Ang II infusion has been reported to increase10 and not to change11,12 NOS3 expression in the whole medulla. This discrepancy raises the likelihood that either Ang II reduces NOS3 expression in some medullary structures while increasing it in others, or Ang II stimulates multiple pathways that have opposing effects on NOS3 expression, and, therefore, the final result changes depending on experimental conditions.

Acutely, Ang II enhances NO and superoxide (O2−) production in the kidney in general13,14 and in the THAL specifically.15–17 In endothelial cells, NO reduces NOS3 activity18 and expression.19 In contrast, O2− not only decreases NO bioavailability in THALs20 but also enhances NOS3 expression in endothelial cells.21 Because the renal medulla has the highest capacity for NO production in the kidney,22 we hypothesized that Ang II decreases NOS3 expression in medullary THALs (mTHALs) via NO and that this reduction is partially mitigated by the Ang II–induced increase in O2−.

Methods

Primary Cultures of mTHALs

All of the protocols involving animals were approved by the Henry Ford Hospital Institutional Animal Care and Use Committee. The

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composition of physiological saline used was (in mmol/L) 130.0 NaCl, 2.5 NaH₂PO₄, 4.0 KCl, 1.2 MgSO₄, 6.0 l-α-alanine, 1.0 trisodium citrate, 5.5 glucose, 2.0 calcium delactate, and 10.0 HEPES. The solution was adjusted to 320±3 mosmol/kg of H₂O with mannitol and was pH 7.4 at room temperature. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass) were maintained on a diet containing 0.4% sodium and 1.0% potassium (Teklad Rodent Diet 8640, Harlan-Teklad). Rats weighing 200 to 250 g were anesthetized with ketamine and xylazine (100 and 20 mg/kg of body weight IP, respectively). mTHAL primary cultures were generated as described previously. Briefly, the abdominal cavity was opened, and the kidneys were flushed with 40 mL of ice-cold 0.1% collagenase (Sigma) and 100 U of heparin in physiological saline via retrograde perfusion of the aorta. Kidneys were removed, and coronal sections were cut. The inner stripe of the outer medulla was minced and digested in 0.1% collagenase (HyClone), and 20 ng/mL of epidermal growth factor (Invitrogen). Cells were plated on collagen-coated inserts (0.4-μm pore size, 4.7-cm² area, Corning Costar) at a concentration of 80 μg of protein per insert and placed in an incubator at 37°C and 95% O₂/5% CO₂. Previously, we found that 92% of cells in primary cultures were THALs, as evidenced by positive Tamm-Horsfall staining. After 40 hours of seeding, cells were treated with either vehicle (DMEM/F-12 medium) or Ang II 0.1, 1.0, 10.0, or 100.0 nmol/L (Calbiochem, EMD) for 24 hours. In experiments involving N⁵-nitro-L-arginine methyl ester hydrochloride (L-NAME, an NOS inhibitor), Tempol (a O₂⁻ dismutase mimetic; Sigma) or carboxy-PTIO (c-PTIO; a NO scavenger; Cayman Chemical), cells were preincubated for 1 hour with the reagent, and then the medium was changed to one containing 100 nmol/L of Ang II plus one of the following: (1) L-NAME; (2) Tempol; (3) c-PTIO; or (4) L-NAME+sperrmine NONOate (NO donor, Cayman Chemical) for 24 hours. All of the treatments were done in the presence of 5% FBS.

**Western Blot Analysis**

Cells were washed, centrifuged again, and resuspended in 1 mL of DMEM/F-12 (Invitrogen), supplemented with 5% heat-inactivated FBS (HyClone), 100 U/mL of penicillin and 100 μg/mL of streptomycin (HyClone), and 20 ng/mL of epidermal growth factor (Invitrogen). Cells were plated on collagen-coated inserts (0.4-μm pore size, 4.7-cm² area, Corning Costar) at a concentration of 80 μg of protein per insert and placed in an incubator at 37°C and 95% O₂/5% CO₂. Previously, we found that 92% of cells in primary cultures were THALs, as evidenced by positive Tamm-Horsfall staining. After 40 hours of seeding, cells were treated with either vehicle (DMEM/F-12 medium) or Ang II 0.1, 1.0, 10.0, or 100.0 nmol/L (Calbiochem, EMD) for 24 hours. In experiments involving N⁵-nitro-L-arginine methyl ester hydrochloride (L-NAME, an NOS inhibitor), Tempol (a O₂⁻ dismutase mimetic; Sigma) or carboxy-PTIO (c-PTIO; a NO scavenger; Cayman Chemical), cells were preincubated for 1 hour with the reagent, and then the medium was changed to one containing 100 nmol/L of Ang II plus one of the following: (1) L-NAME; (2) Tempol; (3) c-PTIO; or (4) L-NAME+sperrmine NONOate (NO donor, Cayman Chemical) for 24 hours. All of the treatments were done in the presence of 5% FBS.

**Statistical Analysis**

Results are expressed as the percentage of control±SE. Data were analyzed by the Henry Ford Hospital Biostatistics and Research Epidemiology Department, with an analysis of slope for concentration-dependent responses and paired t test. In some experiments, ANOVA was used with posthoc testing. When multiple pairwise comparisons were done, a procedure for multiple tests of significance was applied using Hochberg’s significance limits.

**Results**

To test our hypothesis we first studied the effect of Ang II on NOS3 expression in primary cultures of mTHALs. Treatment of mTHALs with 10 and 100 nmol/L of Ang II for 24 hours decreased NOS3 expression by 23±9% (n=6; P<0.05 versus control) and 50±5% (n=7; P<0.001 versus control), respectively (Figure 1). Lower Ang II concentrations (0.1 and 1.0 nmol/L) did not significantly affect NOS3 expression (Δ = −7±10 and Δ = −14±22 versus control, respectively). Acutely, Ang II activates NOS in THALs, and NO negatively regulates NOS3 expression in endothelial cells. Therefore, we next studied whether Ang II decreases NOS3 expression via NOS activation and NO production. First, we tested whether NOS activation is required for the Ang II–induced reduction of NOS3 expression. In these experiments, 100 nmol/L of Ang II alone reduced NOS3 expression by 27±5% (n=5). In contrast, in the presence of L-NAME, a NOS inhibitor, Ang II had no significant effect on NOS3 expression (Δ = −5±8% versus control; n=5; P<0.007 versus Ang II alone; Figure 2). L-NAME alone had no effect on basal NOS3 expression (Δ = −2±8% versus control; n=5). These data indicate that NOS activity is required for Ang II to reduce NOS3 expression.

Next, we tested whether NO, per se, was involved in the Ang II–induced inhibition of NOS3 expression, first by using a NO donor. When cells were treated with Ang II in the presence of L-NAME plus the NO donor spermine NONOate (1 μmol/L), NOS3 expression decreased by 22±6% (n=7; P<0.013 versus L-NAME+NO donor; Figure 3). Similarly, Ang II alone decreased expression by 28±7% (n=7). L-NAME plus spermine NONOate did not have any effect on NOS3 expression in the absence of Ang II (Δ = 2±10%; n=7).
Next, we used c-PTIO, a NO scavenger. In these experiments, Ang II alone reduced NOS3 expression by 33±4% (n=6). In contrast, in the presence of 10 μmol/L of c-PTIO, Ang II had no significant effect on NOS3 expression (Δ=−1±8%, n=6, versus c-PTIO alone; Figure 4). c-PTIO alone did not significantly change NOS3 expression (n=6). Taken together, these data indicate that NO is required for Ang II to reduce NOS3 expression.

Ang II not only increases THAL NO, but also O2− production, which scavenges NO. Consequently, we studied whether scavenging O2− with Tempol25 exacerbated the effect of Ang II on NOS3 expression. Ang II alone reduced NOS3 expression by 29±3% (n=6). Surprisingly, Ang II failed to decrease NOS3 expression in the presence of Tempol (Δ=−3±7%, n=6; P<0.015 versus Ang II). Tempol alone did not affect NOS3 expression (Δ=6±12%; n=6; Figure 5).

The Tempol results could be due to either a reduction in O2−, which is required for Ang II to decrease NOS3, or the generation of hydrogen peroxide (H2O2), which has been reported to enhance NOS3 expression in endothelial cells. We reasoned that, if H2O2 was masking the effect of NO in NOS3 expression, then in the presence of Tempol Ang II should increase NOS3 expression when NO is inhibited. Thus, we incubated cells with or without Ang II in the presence and absence of both l-NAME and Tempol. Ang II alone reduced NOS3 expression by 40±6%. In contrast, Ang II had no effect on NOS3 expression in the presence of l-NAME and Tempol (Δ=2±7% versus l-NAME+Tempol). l-NAME and Tempol did not affect basal NOS3 expression in the absence of Ang II. Taken together, these data indicate that the ability of Tempol to block Ang II–induced inhibition of NOS3 expression is attributed to a reduction in O2− rather than an increase in H2O2.

**Discussion**

Our hypothesis was that Ang II decreases NOS3 expression in mTHALs via enhanced NO production. We used primary cultures of mTHALs to avoid the confounding influences that changes in blood pressure, renal blood flow, tubuloglomerular feedback, and transport in the proximal tubule caused by Ang II infusion may have on mTHAL NOS3 expression. We found that treatment of mTHAL primary cultures with 10 and 100 nmol/L of Ang II for 24 hours decreased NOS3 expression in a concentration-dependent manner. To our knowledge, this is the first time that the chronic effect of Ang II on NOS3 expression in mTHALs has been studied.

The literature concerning the effect of Ang II on renal medullary NOS3 expression is controversial. Decreases, no effect, and increases have been reported. Wickman et al found that, in 2-kidney, 1-clip hypertension, a model of

![Figure 2](image_url)

**Figure 2.** Effect of NOS inhibition on Ang II–induced decrease in NOS3 expression in mTHALs. Top, Representative Western blot. Bottom, Cumulative data from 5 independent measurements. *P<0.007 vs l-NAME+Ang II.

![Figure 3](image_url)

**Figure 3.** Effect of NOS inhibition and addition of exogenous NO on Ang II–induced decrease in NOS3 expression in mTHALs. Top, Representative Western blot. Bottom, Cumulative data from 7 independent measurements. *P<0.013 vs l-NAME+spermine NONOate.

![Figure 4](image_url)

**Figure 4.** Effect of NO scavenging on Ang II–induced decrease in NOS3 expression in mTHALs. Top, Representative Western blot. Bottom, Cumulative data from 6 independent measurements; no significant difference.

![Figure 5](image_url)

**Figure 5.** Effect of O2− dismutation on Ang II–induced decrease in NOS3 expression in mTHALs. Top, Representative Western blot. Bottom, Cumulative data from 6 independent measurements; *P<0.015 vs Tempol+Ang II.
high-circulating Ang II, NOS3 expression is decreased in the outer medulla, the location of mTHALs. This result was observed in both clipped and unclipped kidneys after 42 days of clipping, suggesting that the effect was because of Ang II and not increased renal perfusion pressure. In contrast, 7 days of Ang II infusion at 600 ng·kg⁻¹·min⁻¹ did not affect expression in the outer medulla.¹¹ Infusion for 14 days at ≈280 ng·kg⁻¹·min⁻¹ produced similar results when the whole medulla was studied.¹² Finally, when infused at 200 ng·kg⁻¹·min⁻¹ for 3 days, Ang II increased NOS3 expression in the whole medulla.¹⁰

The apparent discrepancies may be explained by differences in experimental design, including tissue studied, dose of Ang II used, and time of treatment. The medulla is composed of THALs, thin descending and ascending limbs, outer and inner medullary collecting ducts, vasa recta, and interstitial cells. Thus, the disparate results in these studies may simply be because of Ang II having different effects on the various cell types. Support for such a hypothesis comes from data showing that Ang II enhances NOS3 expression in the cortex,¹¹,¹² which is 80% to 90% proximal tubule cells, whereas our data show that it decreases NOS3 expression in mTHALs.

Differences in the doses of Ang II used may also account for the contrasting results. The concentration-response relationship between Ang II and NOS3 expression in the outer medulla may be complex. The effect of Ang II on ion transport²²,³³ and the regulation of NOS3 expression by endothelin-¹ in the THAL is biphasic. We found that 10 and 100 nmol/L of Ang II, similar to concentrations measured in the kidney in vivo,³⁴–³⁶ inhibit NOS3 expression. We also found that 0.1 and 1.0 nmol/L had no effect, but we did not test lower concentrations. It is possible that concentrations of Ang II <0.1 nmol/L stimulate expression as they do transport.³³

Finally, the effect of Ang II on NOS3 expression may be time dependent. In mTHALs, a high-salt diet increases NOS3 expression in a biphasic manner, peaking at 3 days and returning to basal levels at 14 and 28 days.³⁷

In endothelial cells, NO donors decrease NOS3 expression,¹⁹ and, acutely, Ang II increases NO production in the THAL.¹⁵ Thus, we next tested whether Ang II reduces NOS3 expression via an increase in NO through negative feedback in mTHALs. We found that the effect of Ang II on NOS3 expression was blocked by L-NAME. Thus, we studied whether it restores the effect; and scavenging NO with c-PTIO blocked Ang II–induced decreases in NOS3 expression in the outer medulla.¹⁰

Our results show that both NO and O₂⁻ are required for Ang II to inhibit NOS3 expression in mTHALs. O₂⁻ can react with NO to form peroxynitrite (ONOO⁻), suggesting that ONOO⁻ may mediate the actions of Ang II on NOS3. Ang II has been shown to increase tyrosine nitration, a marker of ONOO⁻,⁴⁴,⁴⁵ in endothelial cells,⁴⁶ proximal tubules,⁴⁷ and renal outer medullary tissue.⁴⁸ In bovine aortic endothelial cells, ONOO⁻ decreases NOS3 expression,⁴⁹ whereas in diabetes mellitus, an inverse relationship between NOS3 expression and tyrosine nitration has been delineated.⁵⁰ Moreover, it has been shown that treatment of endothelial cells with low-density lipoprotein and oxidized low-density lipoprotein results in increased ONOO⁻ production and decreased NOS3 expression.⁵¹,⁵² Thus, ONOO⁻ may mediate the effects of Ang II on mTHAL NOS3 expression. However, additional experiments are needed to confirm the involvement of ONOO⁻.

The source of O₂⁻ responsible for Ang II–induced decreases in mTHAL NOS3 expression needs further study. However, the most likely sources are NADPH oxidase and NO itself. Ang II increases O₂⁻ production via NADPH oxidase in the THAL.¹⁷ In addition, Ang II induces NO uncoupling,⁴⁶,⁵³ thereby increasing O₂⁻ production. Thus, it is possible that NOS itself could become the source of both the NO and O₂⁻ required for Ang II to inhibit NOS3 expression. Although we showed that L-NAME blocks the effects of Ang II, and L-NAME could block both NO and O₂⁻ production by NOS,⁵³–⁵⁵ the addition of exogenous NO was sufficient to revert the blockade of Ang II–induced decreases
in NOSS3 caused by 1-NAME. Thus, these data appear to rule out the possibility that uncoupled NOSS3 is the source of $O_2^\cdot$.

**Perspectives**

We conclude that Ang II decreases NOSS3 expression in mTHALs via both NO and $O_2^\cdot$ and, thus, raises the possibility that ONOO$^{-}$ is involved. NO produced by THAL NOSS3 inhibits CI$^-$ reabsorption in this nephron segment. Conversely, Ang II is a pleiotropic hormone that produces vasoconstriction56 and enhances NaCl reabsorption by the kidney.57,58 Decreased NOSS3 expression induced by Ang II may be one of the mechanisms by which this hormone enhances sodium retention. Reduced NOSS3 may result in decreased NO production and bioavailability. This, in turn, may increase NaCl transport and diminish NO diffusion to the vasa recta, leading to impaired medullary blood flow, all of which have been shown to induce hypertension.59,60 However, whether the same response is observed in vivo needs further study. Understanding the uniqueness of the effect of Ang II in each nephron segment and discerning the pathways that lead to an imbalance between pro-oxidant and antioxidant species would allow the design of better drugs for the treatment of hypertension.

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**Disclosures**

None.

**References**


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