Angiotensin II-mediated hypertension impairs nitric oxide-induced NKCC2 inhibition in thick ascending limbs

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Ramseyer VD, Ortiz PA, Carretero OA, Garvin JL. Angiotensin II-mediated hypertension impairs nitric oxide-induced NKCC2 inhibition in thick ascending limbs. Am J Physiol Renal Physiol 310: F748–F754, 2016. First published February 17, 2016; doi:10.1152/ajprenal.00473.2015.—In thick ascending limbs (THALs), nitric oxide (NO) decreases NaCl reabsorption via cGMP-mediated inhibition of Na-K-2Cl cotransporter (NKCC2). In angiotensin (ANG II)-induced hypertension, endothelin-1 (ET-1)-induced NO production by THALs is impaired. However, whether this alters NO’s natriuretic effects and the mechanisms involved are unknown. In other cell types, ANG II augments phosphodiesterase 5 (PDE5)-mediated cGMP degradation. We hypothesized that NO-mediated inhibition of NKCC2 activity and stimulation of cGMP synthesis are blunted by PDE5 in ANG II-induced hypertension. Sprague-Dawley rats were infused with vehicle or ANG II (200 ng·kg−1·min−1) for 5 days. ET-1 reduced NKCC2 activity by 38 ± 13% (P < 0.05) in THALs from vehicle-treated rats but not from ANG II (200 ng·kg−1·min−1) for 5 days. ET-1 reduced NKCC2 activity by 38 ± 13% (P < 0.05) in THALs from vehicle-treated rats but not from ANG II-hypertensive rats (Δ: −9 ± 13%). A NO donor yielded similar results as ET-1. In contrast, dibutyryl-cGMP significantly decreased NKCC2 activity in both vehicle-treated and ANG II-hypertensive rats (control: Δ: −44 ± 15% vs. ANG II: Δ: −41 ± 10%). NO increased cGMP by 2.08 ± 0.36 fmol/μg protein in THALs from vehicle-treated rats but only 1.06 ± 0.25 fmol/μg protein in ANG II-hypertensive rats (P < 0.04). Vardenafil (25 nM), a PDE5 inhibitor, restored NO’s ability to inhibit NKCC2 activity in THALs from ANG II-hypertensive rats (Δ: −60 ± 9%, P < 0.003). Similarly, NO’s stimulation of cGMP was also restored by vardenafil (vehicle-treated: 1.89 ± 0.71 vs. ANG II-hypertensive: 2.02 ± 0.32 fmol/μg protein). PDE5 expression did not differ between vehicle-treated and ANG II-hypertensive rats. We conclude that NO-induced inhibition of NKCC2 and increases in cGMP are blunted in ANG II-hypertensive rats due to PDE5 activation. Defects in the response of THALs to NO may enhance NaCl retention in ANG II-induced hypertension.

cGMP; kidney; sodium transport; phosphodiesterase 5; endothelin-1

THE THICK ASCENDING LIMB of the loop of Henle (THAL) reabsorbs 20 to 30% of the filtered NaCl load and contributes to the generation of the corticomedullary osmotic gradient and blood pressure regulation (12). Na-K-2Cl cotransporter type 2 (NKCC2) activity accounts for most of the transcellular NaCl reabsorption, transporting 80% of Na and 100% of Cl. The importance of NKCC2 in NaCl reabsorption is underscored by the profuse diuresis induced by loop diuretics like furosemide, which inhibit NKCC2 activity (13). In addition to regulating urine concentration, the THAL plays an important role in the maintenance of blood pressure. Defects in THAL transport that diminish NKCC2 activity cause hypotension (16), whereas increased THAL NaCl reabsorption has been found in hypertension (10, 36).

Angiotensin II (ANG II) is a potent vasoconstrictor and anti-natriuretic hormone. Its importance in blood pressure regulation is underscored by the effectiveness of anti-hypertensive drugs that block ANG II production or actions (5, 43, 44) and by the hypertension induced by chronic ANG II infusion (11, 14). ANG II enhances Na reabsorption directly by increasing Na transport in proximal tubules (6), THALs (40, 45), and collecting ducts (29), and indirectly by reducing the responsiveness to some natriuretic stimuli in collecting ducts (19). However, whether ANG II-induced hypertension also affects THAL response to natriuretic stimuli is not known.

In contrast, nitric oxide (NO) plays an important role in the kidney by inhibiting Na reabsorption in proximal tubules (35), THALs (28, 31), and collecting ducts (42) and by increasing renal blood flow (22). In the THAL many autacoids and hormones, such as endothelin-1 (ET-1), ATP, clonidine, and L-arginine, inhibit NKCC2 via NO (17, 27, 30, 31, 39, 41). Furthermore, infusion of subpressor doses of the nitric oxide synthase (NOS) inhibitor L-NAME induces salt-sensitive hypertension (37), indicating that impaired NO production and/or signaling contributes to high blood pressure.

We recently showed that in ANG II-mediated hypertension ET-1-induced NO production by THALs is impaired and that this correlates with reduced NOS3 expression and altered phosphorylation (33). However, whether ET-1- and NO-induced inhibition of NKCC2 is also impaired or whether changes downstream from NO compensate for this defect remain to be elucidated.

NO inhibits NKCC2 activity via cGMP (27), and cGMP can be degraded by phosphodiesterases (PDEs) (8). In smooth muscle cells, PDE5 reduces NO-induced elevations in cGMP (9, 21), and in the kidney PDE5 regulates Na reabsorption in pregnant rats (38). Interestingly, acute ANG II treatment increases PDE5 levels and activity in smooth muscle cells (18) and ANG II-induced hypertension elevates PDE5 activity in cardiomyocytes (23). However, whether PDE5 plays a role in the THAL in ANG II-induced hypertension is not known. Therefore, we hypothesized that NO-induced inhibition of NKCC2 activity and increases in cGMP are blunted in THALs of ANG II-hypertensive rats and this is mediated in part by PDE5.

MATERIALS AND METHODS

Reagents and solutions. Most chemicals and reagents were purchased from Sigma (St. Louis, MO). ET-1 was from Bachem (Bubendorf, Switzerland). The NO donor spermine NONOate was from Caymen Chemical (Ann Arbor, MI). Dibutyryl (db)-cGMP was from

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ENZO Life Sciences (Ann Arbor, MI). Sodium Green was from Invitrogen (Grand Island, NY). Vardenafil was from Bayer AG (Leverkusen, Germany). The CGMP enzyme-immunoassay kit was from Biomedical Technologies (Stoughton, MA). Coomassie Plus protein assay reagent was from Pierce (Rockford, IL). The PDE5A1 antibody was from BD Transduction (San Jose, CA) and the secondary antibody was from GE Healthcare.

Physiological saline contained (in mM) 130 NaCl, 2.5 NaH2PO4, 4 KCl, 1.2 MgSO4, 6 D/L-alanine, 1 trisodium citrate, 5.5 glucose, 2 calcium lactate, and 10 HEPES (pH 7.4 with NaOH). The NaCl-free solution contained the same ingredients except that NaCl and KCl were omitted, mannitol was added in equi-osmolar amounts, and the solution pH was adjusted with KOH.

ANG II-induced hypertension. All protocols involving animals were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. Male Sprague-Dawley rats weighing 90–120 g were fed a standard chow containing 0.4% Na and infused with ANG II at 200 ng·kg⁻¹·min⁻¹ via osmotic mini-pumps (Durect; model 1007D) for 5 days. Mean arterial blood pressure was measured by a femoral arterial catheter in anesthetized rats (90 mg/kg ketamine, 10 mg/kg xylazine) using a pressure transducer and PowerLab software (ADInstruments, Colorado Springs, CO).

Isolation and perfusion of rat THALs. THALs were isolated and perfused as we have done before (17). Once the rat was anesthetized, the abdominal cavity was opened, and the kidneys were bathed in ice-cold saline and removed. Coronal slices were placed in oxygenated physiological saline. THALs were dissected from the outer medulla under a stereomicroscope at 4°C and transferred to a temperature-regulated chamber, where they were perfused using concentric glass pipettes at 37 ± 1°C.

Measurement of NKCC2 activity. NKCC2 activity was measured using methods similar to those we reported (28). Briefly, tubules were perfused at 37°C in a chamber on the stage of an inverted microscope with the NaCl-free solution (to inhibit NKCC2 activity) with 100 μM dimethyl amiloride (to inhibit Na/H exchange) and bathed in physiological saline. Tubules were loaded for 20 min with 2 μM Sodium Green in the bath and washed for 20 min with physiological saline. Dye was excited at 490 nm and fluorescence emission was recorded at >500 nm to measure intracellular Na every 5 s for a 12-min control period. Acquired data were processed using Metafluor (Molecular Devices, Sunnyvale, CA). Then, the luminal solution was switched to physiological saline plus dimethyl amiloride to stimulate NKCC2. Twenty minutes after the test compound was added to the bath and the process was repeated. The first five points were used to calculate the initial rate of fluorescence increase. NKCC2 activity was calculated from the initial rate of change normalized by the fluorescence at the time of the solution switch.

In the experiments using ET-1, 100 μM l-arginine was present in the bath. The NO donor spermine NONOate (100 μM) was prepared 2 min before being added to the bath to minimize degradation. When vardenafil (25 nM) was used to inhibit PDE5, it was added to the bath solution during the wash and was present for the remainder of the experiment.

THAL suspensions. Suspensions were prepared as we reported (32, 34). Briefly, the abdominal cavity was opened, and the kidneys were perfused with 40 ml of ice-cold physiological saline containing 0.1% collagenase (cat. no. C0130, 233 U/mg, Sigma) and 100 U heparin via the aorta. Kidneys were removed, and coronal slices were cut. The inner stripe of the outer medulla was minced and incubated in physiological saline containing 0.1% collagenase for 30 min at 37°C. Every 5 min, the tissue was gently agitated and gassed with 100% oxygen. Tissue was centrifuged at 60 g for 2 min; the pellet was resuspended in cold physiological saline and stirred on ice for 30 min. The suspension was filtered through a 250-μm nylon mesh and centrifuged at 60 g for 2 min. The pellet was washed and centrifuged again to collect THALs.

cGMP AND NKCC2 ACTIVITY IN HYPERTENSION

RESULTS

Rats infused with ANG II had a significantly higher mean arterial blood pressure than vehicle-treated rats (vehicle-treated: 91.5 ± 2.4 mmHg vs. ANG II-hypertensive rats: 106.1 ± 3.4 mmHg, n = 20 and 26, respectively, P < 0.002), demonstrating that infusion of this dose of ANG II for 5 days produces a small but significant increase in blood pressure. Because of our previous findings showing that THAL NO production stimulated by ET-1 is impaired in ANG II-induced hypertension, we first tested whether ET-1-induced NO inhibits NKCC2 activity in THALs from ANG II-hypertensive rats. In THALs from vehicle-treated rats, ET-1 decreased NKCC2 activity by 38 ± 13% (n = 5, P < 0.05; Fig. 1, A and B). However, in ANG II-hypertensive rats, ET-1’s inhibitory effect was blunted (Δ: −9 ± 13%; n = 6; N.S.: Fig. 1, C and D). These data indicate that the ability of ET-1 to decrease NKCC2 activity is impaired in THALs from ANG II-induced hypertensive rats.

The effects of ET-1 could be diminished due to a reduction in the bioavailability of NO or due to decreased NO signaling. Since reactive oxygen species can reduce NO bioavailability, we tested whether scavenging superoxide with tempol could normalize the response to ET-1. However, pretreatment with 100 μM tempol did not restore the ability of ET-1 to decrease NKCC2 activity in THALs from ANG II-hypertensive rats (Δ in NKCC2 activity: +14 ± 19%, not significantly different, n = 5; Fig. 2). These data indicate that the impaired ability of ET-1 to inhibit NKCC2 activity in ANG II-hypertensive rats is not due to acute elevations of superoxide.

We reported that ET-1 acts via NO in THALs. Thus, we next tested whether inhibition of NKCC2 by exogenously added NO
was blunted in ANG II-induced hypertension. In THALs from vehicle-treated rats, treatment with the NO donor spermine NONOate (100 μM) reduced NKCC2 activity by 33 ± 6% (P < 0.003, n = 6; Fig. 3A). In contrast, in THALs from ANG II-hypertensive rats, NKCC2 activity was not significantly decreased by treatment with the NO donor (Δ: +23 ± 14%, n = 6; Fig. 3B). Taken together, these results and the ET-1 data indicate that NO-induced inhibition of NKCC2 is blunted in THALs from ANG II-induced hypertensive rats.

NO inhibits NKCC2 activity via cGMP. To test whether the response to cGMP was reduced in THALs from ANG II-induced hypertensive rats, we measured NKCC2 activity before and after incubation with the cGMP analog db-cGMP (500 μM). In vehicle-treated rats, db-cGMP reduced NKCC2 activity by 44 ± 15% (P < 0.05, n = 5; Fig. 4A). Similarly, in ANG II-hypertensive rats db-cGMP reduced NKCC2 activity by 41 ± 10% (P < 0.03, n = 4; Fig. 4B). These data suggest that the signaling cascade downstream from cGMP is intact in THALs from ANG II-induced hypertensive rats.

We next studied whether NO-induced cGMP levels were diminished in ANG II-hypertensive rats. In THALs from vehicle-treated rats, 100 μM spermine NONOate increased cGMP by 2.08 ± 0.36 fmol/μg protein (n = 6) but only 1.06 ± 0.25 fmol/μg protein in tubules from ANG II-hypertensive rats (n = 8, P < 0.04; Fig. 5). These data indicate that NO-induced increases in cGMP are impaired in THALs from ANG II-induced hypertensive rats.

PDE5 mediates decreases in cGMP in response to ANG II in other cell types. Thus, we tested whether PDE5 was responsible for the aforementioned results. In the presence of the PDE5 inhibitor vardenafil (25 nM), 100 μM spermine NONOate decreased NKCC2 activity by 45 ± 12% in THALs from ANG II-induced hypertensive rats.
vehicle-treated rats ($P < 0.03, n = 4$; Fig. 6A). Interestingly, vardenafil treatment restored the ability of spermine NONOate to decrease NKCC2 activity in tubules from ANG II-induced hypertensive rats ($\Delta: -60 \pm 9\%, P < 0.003, n = 5$; Fig. 6B). Time controls with vardenafil showed no significant effect.

In the presence of vardenafil, 100 $\mu$M spermine NONOate increased cGMP to the same extent in THALs from vehicle-treated and ANG II-hypertensive animals [Fig. 7; vehicle-treated: $1.89 \pm 0.71$ fmol/µg protein ($n = 6$) vs. ANG II-induced hypertensive: $2.02 \pm 0.32$ fmol/µg protein ($n = 8$)]. Basal cGMP levels were no different between vehicle and ANG II-hypertensive rats. These data indicate that in ANG II-induced hypertensive rats PDE5 blunts the ability of NO to inhibit NKCC2 activity by enhancing cGMP degradation.

Finally, to study whether PDE5 expression was different between groups, we performed Western blots. PDE5 expression was not different in THALs from vehicle-treated and ANG II-induced hypertensive rats (PDE5/β-tubulin ratio $= 0.55 \pm 0.07$ in vehicle-treated rats vs. $0.47 \pm 0.11$ in ANG II-hypertensive rats; $n = 5$; Fig. 8).

**DISCUSSION**

We hypothesized that NO’s inhibition of NKCC2 activity and stimulation of cGMP synthesis are blunted via PDE5 in ANG II-induced hypertension. We found that PDE5 reduces NO-induced inhibition of NKCC2 activity and this is likely due to enhanced degradation of cGMP by PDE5 in THALs from ANG II-induced hypertensive rats. This conclusion is based on the following findings: 1) ET-1- and NO-induced inhibition of NKCC2 activity was impaired in THALs from ANG II-induced hypertensive rats; 2) db-cGMP inhibited NKCC2 activity to the same extent in ANG II-induced hypertensive and vehicle-treated rats; 3) NO-induced increases in cGMP were impaired in THALs from ANG II-induced hypertensive rats; and 4) NO-induced decreases in NKCC2 activity and increases in cGMP were restored in THALs from ANG II-induced hypertensive rats.

**Fig. 3.** Effect of NONOate (NO) on NKCC2 activity. NKCC2 activity was measured in isolated and perfused THALs before and after treatment with 100 $\mu$M spermine NONOate. A: vehicle-treated rats ($n = 6$). B: ANG II-induced hypertensive rats ($n = 6$).

**Fig. 4.** Effect of db-cGMP on NKCC2 activity. NKCC2 activity was measured in isolated and perfused THALs before and after treatment with 500 $\mu$M db-cGMP. A: vehicle-treated rats ($n = 5$). B: ANG II-induced hypertensive rats ($n = 4$).

**Fig. 5.** Effect of NONOate (NO) on cGMP levels in THALs from vehicle- and ANG II-induced hypertensive rats. THAL suspensions were incubated in the presence and absence of 100 $\mu$M spermine NONOate for 10 min (vehicle $n = 6$; ANG II $n = 8$). cGMP was measured by enzyme immunoassay.
hypertensive rats by inhibiting PDE5. To our knowledge, this is the first time that impaired NO-induced inhibition of Na reabsorption in THALs has been shown in ANG II-induced hypertension. Moreover, this is the first time that PDE5 has been shown to play a role in NaCl reabsorption in any nephron segment during ANG II-induced hypertension.

We found that ET-1-induced inhibition of THAL NKCC2 activity was reduced in ANG II hypertension. The inability of ET-1 to reduce NKCC2 activity is likely due to two factors: 1) diminished ET-1-induced NO production; and 2) blunted effect of NO. We previously reported that ET-1-induced NO production is blunted in this model (33) and now show that the effects of a NO donor are also reduced. Of note, we did not observe higher absolute values in basal NKCC2 activity in isolated and perfused THALs from rats treated with ANG II when compared with vehicle-treated rats. The reason for this finding is not clear but may be due to different causes. First, the experimental design was aimed to measure NKCC2 activity in response to acute treatments within the same tubule and was not intended to compare NKCC2 activity in different rats which requires special experimental strategies. This specific experimental design is necessary to test for differences between groups because there is high interexperimental variability when measuring NKCC2 activity in isolated and perfused THALs. Additionally, NKCC2 activity was measured under conditions in which NO production was not promoted (except during ET-1 treatment). Thus, differences in NKCC2 activity caused by changes in NO production or effect would not be present. Finally, other signals that are not present in isolated and perfused THALs might be necessary to observe increases in basal NKCC2 activity (3). Regardless of whether basal NKCC2 activity is different in isolated and perfused THALs from vehicle- vs. ANG II-hypertensive rats, our data conclusively demonstrate that NO-mediated inhibition of NKCC2 activity is impaired in this model of hypertension.

The fact that ET-1 has less of an effect on NKCC2 activity in THALs from ANG II-induced hypertensive rats suggests that either bioavailability or NO signaling is reduced. ANG II-induced hypertension increases superoxide production by hypertensive rats by inhibiting PDE5. To our knowledge, this is the first time that impaired NO-induced inhibition of Na reabsorption in THALs has been shown in ANG II-induced hypertension. Moreover, this is the first time that PDE5 has been shown to play a role in NaCl reabsorption in any nephron segment during ANG II-induced hypertension.

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![Graph](https://example.com/graph1.png)

**Fig. 6.** Effect of NO on NKCC2 activity in the presence of the phosphodiesterase 5 (PDE5) inhibitor vardenafil. Effect of 100 μM spermine NONOate on NKCC2 activity in the presence of 25 nM vardenafil in isolated and perfused THALs. A: vehicle-treated rats (n = 4). B: ANG II-induced hypertensive rats (n = 5).

![Graph](https://example.com/graph2.png)

**Fig. 7.** Effect of NO on cGMP levels in the presence of the PDE5 inhibitor vardenafil in THALs from vehicle- and ANG II-induced hypertensive rats. THAL suspensions were preincubated with 25 nM vardenafil for 10 min and then treated with 100 μM spermine NONOate for 10 min (vehicle n = 6; ANG II n = 8). cGMP was measured by enzyme immunoassay.

![Image](https://example.com/image.png)

**Fig. 8.** PDE5 protein levels in THALs from vehicle- and ANG II-induced hypertensive rats. Top: representative Western blot for PDE5 and β-tubulin. Bottom: cumulative data showing PDE5/β-tubulin ratio (n = 5).
this nephron segment (40) and superoxide decreases NO bioavailability by reacting with it to form peroxynitrite (24, 26). Thus, we tested whether elevated superoxide was the cause of the diminished ability of ET-1 to inhibit transport. We found that acutely scavenging these reactive oxygen species did not restore the inhibitory effect of ET-1.

To dissect the mechanism by which ANG II-induced hypertension impairs NO’s ability to reduce NKCC2 activity, we next studied the ability of PDE5 to inhibit NKCC2. This cGMP analog is resistant to hydrolysis by PDEs (46). We found that treatment with db-cGMP inhibited NKCC2 activity to the same extent in THALs from normotensive and hypertensive rats. These data indicate that the signaling cascade downstream from cGMP is intact in ANG II-induced hypertensive rats. Since the effect of cGMP was intact but the ability of NO was diminished in THALs from ANG II-induced hypertensive rats, we next measured the ability of the NO donor to elevate cGMP levels. We found that the NO increased cGMP less in THALs from ANG II-induced hypertensive rats compared with tubules from vehicle-treated rats. These data indicate that the cause of NO’s inability to reduce NKCC2 activity in THALs from ANG II-induced hypertensive animals is due to a failure to raise cGMP levels.

Reduced cGMP levels in response to NO could be caused either by decreased production or enhanced cGMP degradation. Previously, we reported that degradation is elevated in another model of salt-sensitive hypertension (15) so we focused on cGMP degradation. At the present time, there are three known cGMP-specific PDEs (PDE5, PDE6, and PDE9) and five PDEs (PDE1, 2, 3, 10, and 11) with dual specificity (8). PDE5 is present in proximal tubules and collecting ducts (20, 25). Additionally, ANG II increases PDE5 activity in smooth muscle cells (18). Thus, we focused on this isoform.

To show that PDE5 was responsible for blunted NO-induced inhibition of NKCC2, we measured the effects of NO on cGMP levels in the presence of a PDE5 inhibitor. We found that blockade of PDE5 restored NO’s ability to reduce NKCC2 activity and increased cGMP in THALs from ANG II-induced hypertensive rats. These data support the conclusion that PDE5 blunts NO-induced inhibition of cotransport in THALs from ANG II-induced hypertensive rats by reducing cGMP.

Similar to our findings, NO donors only cause natriuresis in pregnant rats in the presence of PDE5 inhibition (38); however, unlike our results in THALs in ANG II hypertension where NO production is also impaired, PDE5 inhibition alone increases natriuresis in pregnant rats. Inhibition of PDE5 in inner medullary collecting ducts from pregnant rats also restores the ability of NO donors to increase cGMP levels to the same extent as in nonpregnant rats (38).

Resistance to NO donors in ANG II hypertension has been reported for other renal tissues. In glomeruli cGMP production in response to sodium nitroprusside was decreased (2). However, the authors attributed the defect to reduced cGMP production rather than enhanced degradation (2).

We used vardenafil to inhibit PDE5 because of its specificity and potency. It has more than 10 times the potency of sildenafil and tadalafil (4). The EC50 for PDE5 is ~0.4 nM and, except for PDE6A and B, is at least 500 times more selective for PDE5 than for any other PDE discovered so far. At the concentration used in this study (25 nM), only PDE5 and PDE6 would be inhibited; however, PDE6 has only been found in the retinal photoreceptors, pineal gland, and in some melanoma cells (8) but not in the kidney. Although our data do not exclude the possibility that PDEs with dual specificity could be involved, PDE5 inhibition restored the ability of NO to increase cGMP levels and inhibit NKCC2 activity in ANG II-induced hypertensive rats to levels similar to those seen in control rats. Therefore, our data strongly suggest that PDE5 is the PDE responsible for enhanced cGMP degradation in THALs from ANG II-induced hypertensive rats.

ANG II-induced hypertension did not increase PDE5 levels in THALs. These data suggest that posttranslational mechanisms may be responsible for enhanced PDE5-mediated cGMP degradation in this model of hypertension. PDE5 activity is stimulated directly by increases in cGMP which binds PDE5’s allosteric N-terminus side and indirectly through protein kinase G- or A-mediated PDE5 phosphorylation (7, 21). In contrast, PDE5 activity can be reduced by protein phosphatase 1 (PP1)-mediated dephosphorylation. PP1 activity in turn can be regulated by interacting with different regulatory subunits, by phosphorylation of those subunits, and by endogenous inhibitors (1).

In conclusion, ANG II-induced hypertension blunts NO-induced inhibition of NKCC2 activity due to enhanced degradation of cGMP by PDE5. The inability of the THAL to respond to this important natriuretic factor may contribute to the enhanced NaCl reabsorption observed in this model of hypertension.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: V.D.R., P.A.O., O.A.C., and J.L.G. conception and design of research; V.D.R. performed experiments; V.D.R., P.A.O., and J.L.G. analyzed data; V.D.R., P.A.O., O.A.C., and J.L.G. interpreted results of experiments; V.D.R. prepared figures; V.D.R. and J.L.G. drafted manuscript; V.D.R., P.A.O., O.A.C., and J.L.G. edited and revised manuscript; V.D.R., P.A.O., O.A.C., and J.L.G. approved final version of manuscript.

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