Gene Transfer of eNOS to the Thick Ascending Limb of eNOS-KO Mice Restores the Effects of L-Arginine on NaCl Absorption

Pablo A. Ortiz, Nancy J. Hong, Ding Wang, Jeffrey L. Garvin

Abstract—The thick ascending limb of the loop of Henle (THAL) plays an essential role in the regulation of sodium and water homeostasis by the kidney. L-Arginine, the substrate for nitric oxide synthase (NOS), decreases NaCl absorption by THALs. We hypothesized that eNOS produces the NO that regulates THAL NaCl transport and that selective expression of eNOS in the THAL of eNOS knockout (−/−) mice would restore the effects of L-arginine on NaCl absorption. eNOS−/− mice were anesthetized, the left kidney was exposed, and the renal interstitium was injected with recombinant adenoviral vectors that expressed green fluorescent protein (GFP) or eNOS driven by the promoter of the Na/K/2Cl cotransporter Ad-NKCC2GFP and Ad-NKCC2eNOS, respectively. In Ad-NKCC2eNOS–transduced kidneys, eNOS expression was detected 7 days after injection but was absent in Ad-NKCC2GFP–transduced kidneys. In THALs from eNOS−/− mice transduced with Ad-NKCC2eNOS, adding L-arginine increased DAF-2DA fluorescence, a measure of NO production, by 9.1±1.1% (P<0.05; n=5), but not in THALs transduced with Ad-NKCC2GFP. In THALs from eNOS−/− mice transduced with Ad-NKCC2eNOS, Cl absorption averaged 85.9±11.8 pmol/min per millimeter. Adding L-arginine (1 mmol/L) to the bath decreased Cl absorption to 59.7±11.0 pmol/min per millimeter (P<0.05; n=6). In THALs transduced with Ad-NKCC2GFP, Cl absorption averaged 96.0±21.0 pmol/min per millimeter. Adding L-arginine to the bath did not significantly affect Cl absorption (100.6±20.6 pmol/min per millimeter; n=4). We concluded that gene transfer of eNOS to the THAL of eNOS−/− mice restores L-arginine–induced inhibition of NaCl transport and NO production. These data indicate that eNOS is essential for the regulation of THAL NaCl transport by NO. (Hypertension. 2003;42[part 2]:674-679.)

Key Words: genes ■ nitric oxide synthase ■ mice ■ sodium

The thick ascending limb of the loop of Henle (THAL) plays an essential role in the maintenance of salt and water homeostasis by the kidney. Defects resulting in increased NaCl absorption by this nephron segment have been implicated in the development of salt-sensitive hypertension,1–3 while defects resulting in decreased NaCl absorption by this nephron segment have been implicated in the development of salt-sensitive hypertension. Thus, NO-induced inhibition of NaCl absorption could be due to any isoform. We have previously reported that L-arginine inhibits NaCl absorption in wild-type but not in eNOS knockout (−/−) mice. Consequently, we hypothesized that eNOS is responsible for both NO production and regulation of NaCl absorption in the THAL, and we studied whether gene transfer of eNOS to the THAL of eNOS−/− mice could restore L-arginine–induced NO production and inhibition of NaCl absorption in this nephron segment. We recently developed a gene transfer technique that allows for in vivo expression of foreign transgen es specifically into the THAL using adenoviruses and a cell-type specific promoter.10 In the present study, we found that gene transfer of eNOS in THALs of eNOS−/− mice restored the inhibitory effect of L-arginine on NaCl absorption as well as NO production in this nephron segment. We concluded that eNOS is essential to the regulation of NaCl absorption by the THAL and is the NOS isoform that mediates NO production in this nephron segment. This gene transfer technique allows us to study the role of eNOS expressed in different nephron segments in whole-kidney function and blood pressure regulation.

Methods

Adenoviral Vectors
Recombinant replication-deficient adenoviruses containing the reporter gene green fluorescent protein (GFP) or endothelial NOS (eNOS) under the control of a Na/K/2Cl (NKCC2) cotransporter promoter (Ad-NKCC2GFP and Ad-NKCC2eNOS, respectively)
were constructed by the University of Iowa Gene Transfer Vector Core. The cDNA for eNOS was kindly provided by Dr William Sessa (Yale University School of Medicine, New Haven, Conn). The NKCC2 promoter consisted of a 1.8-kb segment of the 5′ flanking region of the Nkcc2 gene.

In Vivo Kidney Transduction
eNOS homozygous knockout mice (weight, 20 to 30 g) on a C57BL/6J background were bred in the Henry Ford Hospital animal facility. Mice were fed a diet containing 0.5% sodium (Purina). We used a modified version of in vivo kidney transduction described previously in rats. The depth of the injection was adjusted to match the distance between the capsule and the corticomedullary junction for mice weighing 20 to 30 g. Four 5-μL undiluted virus injections (1 to 1.2×1012 particles/mL) were made with the use of a 30-gauge needle attached to PE-50 tubing connected to a nanoliter syringe pump (Harvard Apparatus) set at 5 μL/min. The needle was inserted perpendicularly to the renal capsule and parallel to the medullary rays. Injections were made along a straight line within the capsule, with each 2 injection points separated by 1 mm.

Western Blot of eNOS Expression
Kidneys transduced with either Ad-NKCC2eNOS or Ad-NKCC2GFP were removed 6 to 8 days after injection. Sections of the outer medulla were excised from coronal slices, minced, and homogenized in 0.5 mL of a buffer containing 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 0.3 mol/L sucrose, and a protease inhibitor cocktail with the use of a glass tissue grinder (6 strokes). Cell debris was removed by centrifugation, and an aliquot of the supernatant from medullary tissue was used to determine protein concentration using a Coomassie protein assay reagent (Pierce); 80 μg of protein was separated out by electrophoresis on an 8% polyacrylamide gel and electrotransferred to a PVDF membrane (Millipore). Nonspecific binding was blocked by incubating the membrane for 1 hour with 5% nonfat dry milk in Tris-buffered saline-Tween (TBS-T). TBS-T contained 50 mmol/L Tris (pH 7.5), 500 mmol/L NaCl, and 0.1% Tween 20. The membrane was incubated for 1 hour with a 1:1000 dilution of monoclonal eNOS antibody (BD Pharmingen). Specific binding was blocked by incubating the membrane for 1 hour with 5% nonfat dry milk in Tris-buffered saline-Tween (TBS-T). TBS-T contained 50 mmol/L Tris (pH 7.5), 500 mmol/L NaCl, and 0.1% Tween 20. The membrane was incubated for 1 hour with a 1:1000 dilution of monoclonal eNOS antibody (BD Pharmingen) and then washed with TBS-T. After 1 hour incubation with a 1:1000 dilution of anti-mouse Ig-HRP-linked antibody (Amersham), the membrane was washed again and eNOS was detected through the use of ECL Western reagents (Amersham). All incubations were performed at room temperature.

Immunofluorescence and Confocal Microscopy
Seven days after viral injection, kidneys transduced with Ad-NKCC2eNOS were preserved by retrograde perfusion of the aorta with 150 mmol/L NaCl to flush the kidney, followed by a 15-minute perfusion with 4% paraformaldehyde in buffer containing 150 mmol/L NaCl and 10 mmol/L sodium phosphate (pH 7.4). Kidneys were stored in 4% formaldehyde until sectioning, at which point they were embedded in paraffin and cut into slices 5 μm thick. Longitudinal and transverse sections of the outer medulla were obtained from different kidneys. To expose antigenic sites, fixed sections were deparaffinized with xylene and then hydrated gradually through 100% ethanol, then 95%, 70%, and finally distilled water. Each lasting for 5 minutes. Slides were air-dried and incubated for 2 hours at room temperature with a 1:100 dilution of an antibody against eNOS and then for 1 hour at room temperature with a 1:400 dilution of secondary antibody (Alexa Fluor 568 goat anti-rabbit IgG, Molecular Probes). Fluorescence was detected with a confocal laser scanning system (Noran Instruments) set at 568-nm excitation with a 605/55 BP filter (exposure time, 35 seconds).

Isolation and Perfusion of Thick Ascending Limbs
Thick ascending limbs were obtained from eNOS−/− mice 6 to 8 days after transduction with either Ad-NKCC2GFP or Ad-NKCC2eNOS. On the day of the experiment, mice were anesthetized with ketamine (100 mg/kg body wt IP) and xylazine (20 mg/kg body wt IP). The abdominal cavity was opened and the left kidney was bathed in ice-cold saline and removed. Coronal slices were placed in oxygenated physiological saline. Thick ascending limbs were dissected from the renal medulla at 4° to 10°C with the use of a stereomicroscope. Medullary thick ascending limbs (ranging from 0.5 to 1.0 mm in length) were transferred to a temperature-regulated chamber and perfused with the use of concentric glass pipettes at 37±1°C as described previously. The composition of the basolateral bath and perfusate (physiological saline) was, in mmol/L: 130 NaCl, 2.5 NaH2PO4, 4 KCl, 1.2 MgSO4, 6 alanine, 1 Na citrate, 5.5 glucose, 2 Ca (lactate)2, 10 HEPES (pH 7.4). Solutions were gassed with compressed air before the experiments. The normal flow rate of the basolateral bath was 0.5 mL/min.

Chloride Absorption
Chloride concentration was measured in samples of the bath, perfusate, and collected fluid by means of an ultramicrofluorometric assay. Net chloride absorption (JCl) was calculated according to the equation JCl=V0C0−C1VL, where C0 is the chloride concentration in the perfusate (mmol/L), C1 is the chloride concentration in the collected fluid (mmol/L), V0 is the perfusion rate (nL/min per millimeter), and VL is the collection rate (nL/min per millimeter).

Measurement of Intracellular NO by DAF-2 DA
Intracellular NO production by THALs was measured as described previously. Briefly, isolated, perfused thick ascending limbs were loaded by adding 2 μmol/L of the fluorescent dye DAF-2 DA (Calbiochem) to the bath for 45 minutes. Tubules were washed for 30 minutes with physiological saline. The dye was excited with an argon laser set at 488 nm and fluorescence emitted by NO-bound dye was measured using a scanning laser confocal microscope (Noran Instruments). Measurements were recorded every once for a 10-minute control period; 1 mmol/L L-arginine was then added to the bath, and 10 minutes later, fluorescence was measured for a 10-minute period.

Statistics
Data are reported as mean±SEM. Differences in means were analyzed by unpaired t test or ANOVA as appropriate. Unpaired t tests were used for post hoc testing.

Results
We have previously shown that in vivo transduction of rodent kidneys with an adenovirus carrying a transgene under the control of the NKCC2 promoter results in highly efficient and specific transduction of THALs. We first tested whether in vivo transduction of kidneys from eNOS−/− mice with Ad-NKCC2eNOS would result in expression of eNOS in the THAL. Seven days after injections of either Ad-NKCC2eNOS or Ad-NKCC2GFP, the left kidney from eNOS−/− mice was removed and expression of eNOS in the outer medulla was studied by Western blot. Figure 1A is a representative blot showing eNOS expression only in outer medulla of transduced kidneys but not in other
tissues. No eNOS was detected in contralateral, nontransduced kidneys.

Since we found that transduction with Ad-NKCC2eNOS resulted in eNOS expression in the THAL, we tested whether the enzyme was functional and could produce NO. For this purpose, we measured L-arginine–induced NO production in isolated perfused THALs from eNOS−/− mice 6 to 8 days after transduction with either Ad-NKCC2eNOS or Ad-NKCC2GFP, using the NO-sensitive fluorescent dye DAF-2DA. We found that in THALs from mice transduced with Ad-NKCC2eNOS, adding L-arginine (1 mmol/L) to the bath increased fluorescence by 9.1 ± 1.1% (P < 0.05; n = 5), indicating an increase in NO production. However, in THALs from eNOS−/− mice transduced with Ad-NKCC2GFP, L-arginine did not significantly increase NO production (0.7 ± 0.5% change in DAF-2DA fluorescence; n = 5) (Figure 2). These data indicate that eNOS is essential for the production of NO induced by L-arginine in the THAL.

We next tested whether expression of eNOS in the THAL of eNOS−/− mice could restore the inhibitory effect of L-arginine on NaCl absorption. Six to 8 days after transduction with Ad-NKCC2eNOS, kidneys were harvested, THALs isolated and perfused, and Cl absorption measured. We found that in THALs from eNOS−/− mice transduced with Ad-NKCC2eNOS, basal Cl absorption averaged 85.9 ± 11.8 pmol/min per millimeter. Adding L-arginine (1 mmol/L) to the bath decreased Cl absorption to 59.7 ± 11.0 pmol/min per millimeter (P < 0.05; n = 6) (Figure 3). Thus, L-arginine inhibited Cl absorption by 31.2 ± 5.9%, suggesting that expression of eNOS restores the effects of L-arginine on the THAL of eNOS−/− mice.

We have previously shown that L-arginine has no effect on Cl absorption by THALs from eNOS−/− mice. However, to provide an adequate control for the transduction studies, we examined the effect of L-arginine on Cl absorption by THALs isolated from eNOS−/− mice 6 to 8 days after transduction with Ad-NKCC2GFP. In THALs from mice transduced with Ad-NKCC2GFP, basal Cl absorption averaged 96.0 ± 21.0 pmol/min per millimeter. Adding L-arginine (1 mmol/L) to the bath did not significantly affect Cl absorption (100.6 ± 20.6 pmol/min per millimeter; n = 4) (Figure 4). These data confirm that THALs from eNOS−/− mice do not respond to L-arginine and also indicate that the gene transfer technique per se does not cause changes in eNOS−/− mice.

Figure 1. A, Representative Western blot of eNOS expression in outer medulla of eNOS−/− mouse kidneys 7 days after transduction with either Ad-NKCC2GFP or Ad-NKCC2eNOS (lane 1: Ad-NKCC2GFP-transduced eNOS−/− mice, lanes 2 and 3: eNOS−/− mice transduced with Ad-NKCC2eNOS). B, Representative confocal fluorescent micrograph showing eNOS expression in THALs in a transverse section of the outer medulla of eNOS−/− mouse kidney transduced with Ad-NKCC2eNOS. Scale bar, 20 μm. C, Transmitted image of section shown in B.

Figure 2. Effect of L-arginine on NO production by THALs from mice transduced with either Ad-NKCC2eNOS or Ad-NKCC2GFP. Seven days after transduction, kidneys were harvested, THALs dissected, and NO production in response to 1 mmol/L L-arginine measured by use of fluorescent dye DAF-2DA (*P < 0.05).
that would account for the effects of L-arginine on kidneys transduced with Ad-NKCC2eNOS.

Discussion

We have recently developed an in vivo gene transfer technique that allows expression of different transgenes specifically in the THAL. We used this genetic approach to test our hypothesis that eNOS is essential for NO production and the regulation of NaCl transport by the THAL. Our data show that transduction with adenoviruses carrying a transgene for eNOS under control of the NKCC2 promoter results in expression of eNOS in the THAL of eNOS−/− mice, which restores L-arginine–induced NO production, whereas THALs from eNOS−/− mice transduced with GFP do not produce NO in response to L-arginine. Expression of eNOS in the THAL of eNOS−/− mice restores L-arginine-induced inhibition of NaCl absorption, whereas THALs from eNOS−/− mice transduced with GFP do not respond to L-arginine. Taken together, these data suggest that eNOS is the NOS isoform that mediates NO production and regulates THAL transport. To our knowledge, these data show for the first time that a phenotype caused by genetic deletion of eNOS can be restored by cell type–specific expression of an eNOS transgene in the kidney.

NO is an important regulator of NaCl and water absorption by the kidney. We have previously shown that endogenous NO formed from L-arginine inhibits NaCl absorption by the THAL. However, we did not know which specific NOS isoform mediates NO production. We and others have found that all three known NOS isoforms can be expressed in the THAL; however, given that regulation of expression and activity is different for each isoform, it is important to know which one contributes the most to regulation of transport. To determine which NOS isoform regulates NaCl transport in the THAL, we studied the effects of L-arginine on Cl absorption by THALs isolated from iNOS, nNOS, and eNOS−/− mouse kidneys. We found that in THALs from control (C57BL/6J), iNOS−/−, and nNOS−/− mice, L-arginine inhibited Cl absorption. In THALs from eNOS−/− mice, L-arginine failed to inhibit Cl absorption, whereas a chemical NO donor did. These data provided strong evidence that eNOS mediates both NO production and the effects of L-arginine on Cl absorption in this nephron segment. Since an NO donor effectively decreased Cl transport, these data also demonstrated that the signaling cascade beyond NO production was intact in THALs from eNOS−/− mice. However, it is possible that the protein machinery needed for L-arginine to induce NO production is somehow absent in THALs of eNOS−/− mice. To make sure that eNOS is the NOS isoform that mediates NO production and inhibition of transport in the THAL, we studied whether the selective expression of eNOS in THALs of eNOS−/− mice would restore the effects of L-arginine on Cl absorption and NO production.

Transient gene transfer to kidney tubules has not been successful in the past. We recently developed an in vivo gene transfer technique that allows highly efficient and specific expression of foreign transgenes into the THAL. This technique is based on the use of adenovirus gene transfer techniques but takes advantage of a cell type–specific promoter to drive expression of a transgene in the transduced tissue. Because previous data have shown that expression of GFP in transduced THALs is maximal 7 days after transduction, all studies were conducted 6 to 8 days after injections. We first tested whether we could express eNOS in THALs of eNOS−/− mice by transducing the left kidney with a gene construct coding for eNOS under control of the Na/K/2Cl cotransporter (NKCC2) promoter. As analyzed by Western blot, we found that this procedure resulted in easily detectable levels of eNOS expression in the outer medulla of eNOS−/− mice. Although we and others have previously shown that the NKCC2 promoter is specific for the THAL, we further examined eNOS...
expression in the outer medulla of transduced and non-transduced kidneys by immunofluorescence and confocal microscopy. As detected with an eNOS-specific antibody, fluorescence was only observed in THALs from transduced kidneys, whereas no signal was detected in noninjected contralateral kidneys. Taken together, these data suggest that in vivo transduction of eNOS−/− mouse kidneys results in eNOS expression in the THAL.

To make sure that heterologous expression of eNOS results in functional enzymatic activity, we studied the effects of L-arginine on NO production in THALs isolated from eNOS−/− mice after 7 days of transduction with Ad-NKCC2eNOS or Ad-NKCC2GFP. We found that in THALs expressing eNOS, adding L-arginine to the bath significantly increased NO production, whereas in GFP-transduced THALs, it did not. The magnitude of the NO response caused by L-arginine in transduced mouse THALs was similar to that observed in rat THALs as reported previously using the same fluorescent technique to measure NO. These data indicate that heterologous expression of eNOS results in a fully active enzyme that is able to produce NO. Because adenoviral infection induces inflammation in some tissues, it is possible that adenovirally mediated transduction may result in inflammation and increased iNOS protein expression in the renal medulla. However, when we studied the effects of L-arginine on NO production by THALs from eNOS−/− mice transduced with Ad-NKCC2eNOS, we found that L-arginine did not affect it. Thus, it is not likely that the transduction itself induces either iNOS or nNOS expression that could mediate the effects of L-arginine on NO production.

We next tested whether eNOS expression in THALs from eNOS−/− mice could restore the effects of L-arginine on Cl absorption. We found that adding L-arginine to the bath significantly inhibited Cl absorption in THALs isolated from eNOS−/− mice transduced with Ad-NKCC2eNOS. These data indicate that eNOS is the NOS isoform that mediates the effect of L-arginine on NaCl absorption by the THAL and that the upstream protein machinery necessary for L-arginine to stimulate eNOS in the THAL of eNOS−/− mice is intact. The inhibition of Cl absorption caused by L-arginine in THALs from eNOS−/− transduced with Ad-NKCC2eNOS was of a similar magnitude to that observed in wild-type mice, suggesting that the expression levels of eNOS achieved by viral transduction with our technique are physiological.

Conscious and anesthetized eNOS−/− mice have increased blood pressure. It is frequently assumed that the hypertension in these mice is due to increased peripheral vascular resistance caused by diminished endothelium-derived NO. However, this explanation is not supported by the current literature. It has been shown that in all resistance arteries studied, with the exception of the pulmonary circulation, agonist-induced vasodilation is compensated for by other mechanisms such as prostaglandins or upregulation of other NOS isoforms. However, our data indicate that genetic deletion of eNOS is not compensated for in the THAL by other NOS isoforms. Since we have shown that eNOS-derived NO decreases NaCl absorption, it is possible that part of the hypertension observed in eNOS−/− mice is due to increased salt and fluid absorption by the kidney.

**Perspectives**

Our results show that gene transfer of eNOS to the THAL of eNOS−/− mice restores the effects of L-arginine on NaCl absorption and NO production in this nephron segment. Thus this approach may be used to determine whether eNOS in the THAL plays a role in overall regulation of blood pressure in eNOS−/− mice and in other animal models of hypertension. The gene transfer method used in this study can be adapted to a wide variety of animal studies and could provide an important tool for investigators interested in dissecting the molecular mechanisms involved in regulation of nephron transport. Although this is an exciting tool, this technique is not likely to be used for human gene therapy in its current form because of the transient gene expression observed and the impossibility of repeated injections of adenoviral vectors.

Overall, we found that gene transfer of eNOS specifically to the THAL reversed the phenotype caused by genetic deletion of eNOS in this cell type. We conclude that eNOS is the NOS isoform that mediates the effects of L-arginine on NaCl absorption and NO production by the THAL.

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**References**

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