Expression of Heme Oxygenase-1 in Thick Ascending Loop of Henle Attenuates Angiotensin II-Dependent Hypertension

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ABSTRACT

Kidney-specific induction of heme oxygenase-1 (HO-1) attenuates the development of angiotensin II (Ang II)-dependent hypertension, but the relative contribution of vascular versus tubular induction of HO-1 is unknown. To determine the specific contribution of thick ascending loop of Henle (TALH)-derived HO-1, we generated a transgenic mouse in which the uromodulin promoter controlled expression of human HO-1. Quantitative RT-PCR and confocal microscopy confirmed successful localization of the HO-1 transgene to TALH tubule segments. Medullary HO activity, but not cortical HO activity, was significantly higher in transgenic mice than control mice. Enhanced TALH HO-1 attenuated the hypertension induced by Ang II delivered by an osmotic minipump for 10 days (139±3 versus 153±2 mmHg in the transgenic and control mice, respectively; P<0.05). The lower blood pressure in transgenic mice associated with a 60% decrease in medullary NKCC2 transporter expression determined by Western blot. Transgenic mice also exhibited a 36% decrease in ouabain-sensitive sodium reabsorption and a significantly attenuated response to furosemide in isolated TALH segments. In summary, these results show that increased levels of HO-1 in the TALH can lower blood pressure by a mechanism that may include alterations in NKCC2-dependent sodium reabsorption.


Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme to biliverdin, during which both carbon monoxide (CO) gas and free iron are released. Biliverdin is then reduced to bilirubin by the ubiquitous enzyme biliverdin reductase. There are two major isoforms of HO that are responsible for the breakdown of heme, HO-1 and HO-2. HO-2 is the constitutively expressed isoform, whereas HO-1 is inducible by a wide variety of stimuli, including hypoxia, metals, ischemia, and oxidative stress. In the kidney, both isoforms of HO are present, with the highest level of expression of both isoforms in the renal medulla under basal conditions.1,2

HO enzymes are not only expressed in the renal medulla, but their metabolites, CO and bilirubin, play an important role in the regulation of both renal vascular and tubular function. Renal medullary infusion of the general HO inhibitor, zinc deuteroporphyrin 2,4-bis glycol, results in a significant decrease in renal medullary blood flow.1 In the renal tubules, increases in perfusion pressure increase CO levels, and inhibition of HO activity results in significant attenuation of pressure natriuresis and development of salt-sensitive hypertension.3

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These data suggest an important role for HO and its metabolites in the regulation of both renal function and blood pressure.

The important antihypertensive role for systemic HO-1 induction has been shown in several different experimental models of hypertension in which HO-1 was induced either chemically or genetically with viral vectors. Recently, we reported that kidney-specific induction of HO-1 by renal medullary interstitial infusion of cobalt protoporphyrin attenuated the development of angiotension II (Ang II)-dependent hypertension. Although that work was the first to report that renal-specific induction of HO-1 could attenuate Ang II-dependent hypertension, the importance of vascular versus tubular induction of HO-1 could not be distinguished in this model. To determine the relative importance of tubular induction of HO-1 in thick ascending loop of Henle (TALH) to the attenuation of Ang II-dependent hypertension, we generated a novel transgenic mouse model in which human HO-1 was targeted to TALH cells using the uromodulin (Tamm-Horsfall protein [THP]) promoter (TALH-HHO1). We then used this novel transgenic mouse model to determine the specific effect of increased TALH HO-1 levels on the development of Ang II-dependent hypertension.

RESULTS

Gene Expression, Protein Levels, and HO Activity in TALH-HHO1 Transgenic Mice

To determine if the human uromodulin promoter was able to direct TALH-specific expression of the human HO-1 cDNA, reverse transcription (RT) -PCR was performed for the human HO-1 cDNA in microdissected nephron segments obtained from transgenic TALH-HHO1 mice. A strong band was detected in microdissected TALH segments compared with isolated proximal tubule and medullary collecting duct segments (Figure 1A). Western blot analysis of HO-1 protein levels showed a high level of HO-1 protein samples obtained from the entire renal medulla (not including the papilla) of transgenic TALH-HHO1 mice. The level of HO-1 protein was also elevated in the cortex of transgenic TALH-HHO1 mice compared with nontransgenic mice. The level of HO-1 protein was also elevated in the cortex of transgenic TALH-HHO1 mice compared with nontransgenic mice (Figure 1B). HO activity was significantly (P<0.01) increased by threefold in samples obtained from the entire medulla of transgenic TALH-HHO1 mice; however, no differences in cortical HO activity were detected between transgenic TALH-HHO1 and nontransgenic control mice (Figure 1B). HO activity was significantly (P<0.01) increased by threefold in samples obtained from the entire medulla of transgenic TALH-HHO1 mice; however, no differences in cortical HO activity were detected between transgenic TALH-HHO1 and nontransgenic control mice (Figure 1C). Expression of the human HO-1 isoform in the TALH did not result in any overt morphologic changes in the kidney or increased levels of inflammatory marker proteins such as IL1-β or TNF-α; however, it did result in increased levels of IL-6 protein in the medulla of TALH-HHO1 mice (data not shown).

Colocalization of Human HO-1 with Endogenous THP in the Medulla of TALH-HHO1 Transgenic Mice

TALH cell-specific expression of human HO-1 was next confirmed by immunofluorescent colocalization of HO-1 with endogenous THP, which is a specific marker of TALH tubules. Nontransgenic mice exhibited little HO-1 immunofluorescence in the medulla and no colocalization of HO-1 with endogenous THP (Figure 2, A–C). Transgenic TALH-HHO1 mice exhibited significant HO-1 immunofluorescence and colocalization with THP in the medulla (Figure 2, D–F).

Figure 1. Molecular and biochemical characterization of TALH-HHO1 transgenic mice. (A) Representative RT-PCR for human HO-1 from isolated proximal tubule (PT), TALH, and medullary collecting duct (MCD) tubules. Reactions were performed in the presence or absence of RT. (B) Representative Western blots of HO-1 and HO-2 in renal medulla and cortex of nontransgenic (NT) and transgenic (T) TALH-HHO1 mice (n=3). (C) Heme oxygenase assay from the medulla and cortex of NT and T TALH-HHO1 mice (n=4). *Significant difference from NT mice (P<0.05).

Figure 2. Representative immunofluorescent colocalization of human HO-1 with endogenous THP in the medulla of TALH-HHO1 mice. (A–C) Immunofluorescence for HO-1 and THP and the merged image in NT mice. (D–F) Immunofluorescence for HO-1 and THP and the merged image in T TALH-HHO1 mice. Colocalization of HO-1 with THP was observed in the medulla of T TALH-HHO1 but not NT mice.
endogenous THP in the medulla (Figure 2, D–F). These results show TALH-specific localization of HO-1 protein in the medulla of TALH-HHO1 transgenic mice.

### TALH-Specific Expression of Human HO-1 Attenuates Ang II-Dependent Hypertension

Mean arterial pressure (MAP) under basal conditions was similar in nontransgenic and TALH-HHO1 transgenic mice and averaged 116±3 versus 119±2 mmHg in each group, respectively. MAP was significantly increased (P<0.05) in both nontransgenic and transgenic TALH-HHO1 mice in response to Ang II infusion (Figure 3). However, MAP in response to Ang II infusion was significantly higher (P<0.05) in nontransgenic compared with TALH-HHO1 transgenic mice, averaging 153±2 versus 139±3 mmHg in each group, respectively (Figure 3).

### TALH-HHO1 Mice Exhibit Reduced Ouabain-Sensitive $^{86}\text{Rb}^+$ Uptake and Attenuated Response to Furosemide in Isolated TALH Tubule Segments

Sodium transport in isolated TALH tubule from transgenic and nontransgenic TALH-HHO1 mice was determined by ouabain-sensitive Rb$^+$ uptake. Ouabain-sensitive Rb$^+$ uptake was significantly (P<0.05) reduced in TALH-HHO1 transgenic mice compared with nontransgenic mice, averaging 75±3% of the uptake exhibited in control, nontransgenic mice (Figure 4A). The response to furosemide was also evaluated in isolated TALH tubules from transgenic and nontransgenic TALH-HHO1 mice by Rb$^+$ uptake. In nontransgenic mice, furosemide treatment resulted in a decrease in sodium reabsorption as measured by Rb$^+$ uptake of 30+3% compared with control tubules. However, tubules from transgenic TALH-HHO1 mice sodium reabsorption after furosemide treatment only decreased by 18±1% compared with tubules from untreated TALH-HHO1 mice (Figure 4B).

### DISCUSSION

Systemic HO-1 induction has been consistently reported to lower blood pressure in several models of hypertension. Despite these results, the mechanism by which systemic induction of HO-1 lowers blood pressure is not known. We have previously shown that kidney-specific induction of HO-1 through renal medullary interstitial infusion of cobalt protoporphyrin attenuates the development of Ang II-dependent hypertension. However, we were unable to determine the relative importance of HO-1 induction in different tubule segments, such as the TALH or medullary collecting duct, or the effect of renal medullary vascular induction of HO-1 on the prevention of Ang II-dependent hypertension in the previous model. In the present study, we have used a novel transgenic approach to specifically increase HO-1 levels in the TALH cells in the kidney and examine its effect on the development of Ang II-dependent hypertension and sodium transport in the TALH. TALH-specific induction of HO-1 was able to attenuate the development of Ang II-dependent hypertension by ~15 mmHg compared with nontransgenic mice. The effect of TALH-specific induction of HO-1 on the development of Ang II-dependent hypertension was substantially less than induction observed in our previous study in which HO-1 was
induced throughout the entire renal medulla. This observation suggests that HO-1 induction in other tubular or vascular cells in the renal medulla contributes to the marked attenuation in blood pressure observed in our previous study.

TALH-specific induction of HO-1 in our TALH-HHO1 transgenic mice was also associated with a decrease in sodium transport as measured by ouabain-sensitive Rb⁺ uptake. The effect of TALH-specific induction of HO-1 on sodium transport in the TALH is supported by the observation that renal medullary inhibition of HO-1 was associated with increases in sodium and water reabsorption and development of salt-sensitive hypertension. In contrast, previous studies have shown that the HO metabolite, carbon monoxide, enhanced sodium chloride reabsorption in isolated TALH tubules by stimulation of the apical 70-pS K⁺ channel.

One potential mechanism by which induction of HO-1 in TALH can decrease sodium transport is through antioxidant actions. Previous studies have shown that in vitro induction of HO-1 in primary cultures of TALH cells attenuates oxidative stress caused by Ang II. Induction of HO-1 in TALH cells results in the increased production of both bilirubin and CO, and both of which can attenuate Ang II-mediated superoxide production. The attenuation of superoxide production in TALH tubules is significant, because several studies have shown that increases in superoxide can stimulate sodium reabsorption in the TALH both directly and by suppression of nitric oxide (NO) formation. Another potential mechanism by which the antioxidant actions of HO-1 induction in TALH cells may lower blood pressure in Ang II-dependent hypertension is modification of the NO crosstalk between tubular and vascular structures to regulate blood flow in the renal medulla. NO produced in the TALH can diffuse into surrounding vasa recta capillaries to serve as a buffer against vasoconstrictors such as Ang II. Ang II-mediated increases in TALH superoxide production results in an attenuation of NO release, which decreases medullary blood flow and increases sodium reabsorption. It has previously been reported that superoxide-mediated decrease in NO crosstalk contributes to the decreased medullary

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**Figure 4.** Sodium transport in isolated TALH tubules from T and NT mice. (A) Oubain-sensitive ⁸⁶Rb⁺ uptake in isolated TALH tubules from control (NT) and T TALH-HHO1 mice (n=3). (B) Effect of furosemide to inhibit ⁸⁶Rb⁺ uptake in isolated TALH tubules from control (NT) and T TALH-HHO1 mice (n=3). Results are expressed as percent inhibition of transport exhibited in each genotype. *Significant difference from NT mice (P<0.05).

**Figure 5.** Levels of NKCC2, ROMK, Na/K ATPase, and NHE3 protein in the medulla of NT and T TALH-HHO1 mice. (A) Representative Western blot of medullary protein for NKCC2, ROMK, Na/K ATPase, NHE3, and β-actin. (B) Graphical representation of protein levels of NKCC2 normalized to β-actin (n=3). (C) Graphical representation of protein levels of ROMK normalized to β-actin (n=3). (D) Graphical representation of protein levels of Na/K ATPase normalized to β-actin (n=3). (E) Graphical representation of protein levels of NHE3 normalized to β-actin (n=3). *Significant difference from NT mice (P<0.05).
blood flow observed in the hypertensive Dahl salt-sensitive rat. Enhanced production of bilirubin and CO in the TALH of TALH-HHO1 transgenic mice would act to attenuate superoxide levels and maintain NO release from the TALH, which would then act to preserve renal blood flow and enhance sodium excretion. It is also possible that, like NO, enhanced production of CO in the TALH cells of TALH-HHO1 transgenic mice could also diffuse to the vasa recta to help maintain renal medullary blood flow in transgenic mice infused with Ang II.

The role of NO and CO in crosstalk in TALH-HHO1 transgenic mice requires additional detailed examination in future studies.

The relationship between HO-1 and Ang II in the kidney is complex. Studies in the rat have shown that Ang II infusion induces HO-1 mRNA and protein in the kidney. Additional studies have shown an increase in CO generated from the kidney of Ang II-infused rats. Although the precise mechanism of induction of HO-1 protein and activity by Ang II is not known, increases in reactive oxygen species as well as activation of protein kinase C may be responsible for the induction of HO-1 by Ang II in the rat kidney. However, the increase in renal HO activity and protein by Ang II may be species-specific. Several studies in the mouse have failed to show any increase in renal HO activity or HO-1 protein after Ang II treatment. Therefore, it is unlikely that transgenic overexpression of HO-1 in the TALH amplifies any endogenous increase in HO activity to overcome the hypertensive effect of Ang II in the mouse. However, it is possible that exogenous expression of HO-1 in TALH of the rat kidney could have a greater effect on blood pressure in response to Ang II hypertension through additional amplification of HO activity that occurs with Ang II infusion in the rat kidney.

The levels of NKCC2 transporter were significantly reduced in the medulla of TALH-HHO1 transgenic mice compared with nontransgenic mice. TALH-HHO1 mice also displayed an attenuated response of furosemide to block sodium transport in isolated TALH tubules. The decrease in NKCC2 levels may explain the decrease in overall sodium reabsorption as well as the decrease response to furosemide observed in TALH tubules isolated from the TALH-HHO1 transgenic mice. However, treatment of Ang II-infused mice with furosemide resulted in a greater decrease in blood pressure compared with the response in TALH-HHO1 mice. This finding would suggest that, although HO and its metabolites can affect the levels of NKCC2 in the TALH, they may not inhibit NKCC2 activity to the same degree as furosemide and therefore, do not lower blood pressure to a similar degree. Previous studies have reported that increases in cyclic guanosine monophosphate (cGMP) can decrease surface NKCC2 levels in the TALH acting through cGMP-stimulated phosphodiesterase 2. Because HO-1 induction has been shown to increase cGMP levels through generation of CO, it is possible that induction of HO-1 in TALH cells attenuates NKCC2 through CO-mediated increases in cGMP, resulting in increased degradation of the protein. However, measurement of NKCC2 protein in the apical membrane of the TALH in TALH-HHO1 mice by surface biotinylation was not determined and is needed to fully test this hypothesis.

The role of Ang II in the regulation of sodium reabsorption in the TALH is an area that has been extensively studied and in which there remains controversy. In isolated TALH tubules, low concentrations of Ang II (10⁻¹¹ M) result in the stimulation of Rb⁺ uptake, whereas higher concentrations of Ang II (10⁻⁶ M) were found to inhibit Rb⁺ uptake. Studies performed in isolated perfused TALH tubules found that incubation with 10⁻⁸ M Ang II resulted in decreases in transepithelial net chloride absorption (JCl) as well as transepithelial voltage (Vte). However, recent studies in Ang II-hypertensive rats have shown enhanced thick ascending limb sodium transport-related oxygen consumption mediated through increases in protein kinase C α-activity. These results would suggest an increase in TALH sodium transport by Ang II at high levels.

The TALH serves an important role in the regulation of Na⁺ reabsorption and arterial blood pressure. There are several factors that play an important role in modulating Na⁺ reabsorption in this nephron segment. Factors like superoxide anion, dopamine, vasopressin, and prostaglandins can increase Na⁺ reabsorption, whereas NO and 20-HETE act to decrease Na⁺ reabsorption in the TALH. However, the effect of specific manipulation of these factors in the TALH on blood pressure remains undefined. The results of the present study show that increases in HO-1 expression in the TALH can attenuate sodium reabsorption in the TALH through a mechanism that includes decreased levels of NKCC2 protein. Our results also show that TALH-specific induction of HO-1 can lower blood pressure in chronic Ang II hypertension.

These results strengthen the possibility of developing novel antihypertensive therapies that exclusively induce HO-1 in TALH cells in the kidney. Additional studies are required to determine whether the actions of HO-1 induction in the TALH

Figure 6. Response to Ang II-treated mice to furosemide (40 mg/kg) treatment. Mice were treated with furosemide for 5 consecutive days after implantation of osmotic minipumps. Blood pressures were measured in conscious, freely moving mice over the last 3 days of treatment. Significant difference from Ang II-treated mice (P<0.05).
act solely through tubular mechanisms or through vascular mechanisms as well to lower blood pressure in hypertension.

CONCISE METHODS

Generation of TALH-HHO1 Transgenic Mice
TALH-HHO1 transgenic mice were generated by creation of a transgenic construct in which the human HO-1 cDNA was placed under the transcriptional control of the human uromodulin promoter. The human HO-1 cDNA along with a poly-A tail was PCR-amplified to contain NarI and SalI ends. The resultant PCR product was cloned into the pcDNA3Zero vector (Invitrogen, Carlsbad, CA) to produce the plasmid pCR4Zero-HHO1+pa. The human uromodulin promoter was contained in the vector pHLLS15 that was provided by Dr. Serguei Soukharev (American Red Cross, Rockville, MD). pHLLS15 was digested with Clal and SalI to remove the lacZ gene and then ligated to the NarI/SalI human HO-1 cDNA to create the plasmid pHLLS-HHO1. The TALH-HHO1 construct was isolated from the plasmid vector by digestion with BssHI and then purified and injected into fertilized mouse embryos using established transgenic techniques. TALH-HHO1 mice were derived on a mixed B6SJL genetic background and bred back to C57BL/6J mice for greater than 10 generations.

Ang II Hypertension, Furosemide Treatment, and Blood Pressure Measurement
Ang II was delivered through implantation of osmotic minipumps delivering Ang II at a rate of 1 μg/kg per minute as previously described. Mice were treated daily for 5 days with furosemide (40 mg/kg) intraperitoneal injection 5 days after implantation of the Ang II-containing minipumps. Blood pressure was directly measured by micropipette catheters implanted into the carotid artery using aseptic surgical techniques as previously described. Surgery was performed 1 day after initiation of Ang II infusions. The data are presented as the average of the individual daily recordings over this time period.

RT-PCR from Isolated Nephron Segments
RT-PCR for human HO-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed on isolated proximal tubule, TALH, and medullary cortical collecting duct cells isolated as previously described. The kidneys were immediately removed, and the cortex and outer medulla were separated under a dissecting microscope. The tissues were then digested in a dissection solution consisting of wash solution for RNA isolation. RNA was isolated using a commercially available kit specifically designed for RT-PCR according to manufacturer’s guidelines (RNAlater; Ambion, Austin, TX). The PCR reactions for human HO-1 were cycled as 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute. A total of 35 cycles was performed. PCR primers for the HHO-1 were rthHO1+5′-CAGG-CAGAGATGCTGAGTTC and rthHO1–5′-GATGTTGACAG-GAAGCAGCAT. The primers amplified a 543 bp fragment. As a positive control, GAPDH was amplified. The PCR primers for GAPDH were GAPDH+5′-AGAAGGTGTTGAAGCAGCAT and GAPDH–5′-GATGTTATTCGAAGAGAATGGGA. The primers amplified a 405 bp fragment. PCR products were separated on 0.8% DNA Agar (Midwest Scientific, St. Louis, MO) gels. Gels were visualized under ultraviolet illumination, and images were captured using a gel documentation system with software supplied by the manufacturer (Gel Doc 2000; Bio-Rad, Hercules, CA).

Western Blots
Western Blots were performed on lysates prepared from the entire medulla without the papilla or cortex prepared by homogenization in 250 mM sucrose, 10 mM KPO4, 1 mM EDTA, and 0.1 mM PMSF (pH 7.7) in the presence of protease inhibitors (2 μg/ml aprotinin, leupeptin, and pepstatin). The homogenates were then centrifuged at 3,000 × g for 15 minutes at 4°C, and the supernatants were collected. Samples of 50 μg protein were boiled in Laemmli sample buffer (Bio-Rad, Hercules, CA) for 5 minutes, electrophoresed on 10% or 12% SDS polyacrylamide gels, and blotted onto nitrocellulose membrane. Membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) for 2 hours at room temperature and then incubated with primary antibodies. HO-1 was detected with a mouse anti-HO-1 monoclonal antibody (1:2000; StressGen, Vancouver, Canada). NkCC2 was detected with a rabbit polyclonal antibody (1:1000; Aviva Systems Biology, San Diego, CA). ROMK was detected using a chicken antibody (1:2000; Rockland, Gilbertsville, PA) and IRDye 800 goat anti-mouse IgG (1:5000; Rockland, Gilbertsville, PA) and IRDye 800 goat anti-mouse antibody (1:2000; Rockland, Gilbertsville, PA) and IRDye 800 goat anti-mouse antibody (1:2000; Rockland, Gilbertsville, PA) and IRDye 800 goat anti-mouse antibody (1:2000; Rockland, Gilbertsville, PA). Membranes were incubated in primary antibodies overnight at 4°C. The membranes were then incubated with Alex 680 goat anti-mouse, rabbit, or chicken IgG (1:5000; Invitrogen, Carlsbad, CA) and IRDye 800 goat anti-mouse or rabbit IgG (1:5000; Rockland, Gilbertsville, PA) for 1 hour at room temperature. The membranes were then visualized using an Odyssey infrared imager (Li-COR, Lincoln, NE) that allows for the simultaneous detection of two proteins. Densitometry analysis was performed using Odyssey software (LI-COR, Lincoln, NE). Protein levels are expressed as a ratio to β-actin for each sample.
Immunofluorescence
Immunofluorescence for HO-1 and THP was performed on formalin-fixed tissue samples. Sections of renal medulla (20 μm) were cut and then rinsed in PBS, and they were blocked in 5% normal donkey serum for 1 hour at 4°C. Sections were then incubated with either HO-1 or THP antibody in 5% normal donkey serum overnight at 4°C and then rinsed in PBS. HO-1 was detected using a rabbit anti-HO-1 antibody (1:500; StressGen), and THP was detected using a mouse anti-THP antibody (1:500; Accurate Chemical & Scientific Corp, Westbury, NY). Antibody labeling was visualized using fluorescent-labeled secondary antibodies. Primary antibodies were incubated with fluorophore-conjugated species-specific secondary antibodies by incubating for 1 hour in 5% normal donkey serum. Secondary antibodies consisted of a donkey anti-rabbit Alexa488 (1:1000; Invitrogen) and a donkey anti-mouse Cy3 (1:200; Jackson Immunoresearch, West Grove, PA). After a final rinse in PBS, samples were covered with Gel Mount mounting media and cover-slipped before imaging. All samples were examined using fluorescent confocal microscopy (Lica Microsystems, Exton, PA).

HO Assay
HO assay was performed on total renal medullary and cortical lysates as previously described.8,10 Tissues were homogenized in 250 mM sucrose, 10 mM KPO4, 1 mM EDTA, and 0.1 mM PMSF (pH 7.7) in the presence of protease inhibitors (2 μg/ml aprotinin, leupeptin, and pepstatin). The homogenate was then centrifuged at 3,000 × g for 15 minutes at 4°C, and the supernatant was collected and further centrifuged at 10,000 × g for 15 minutes at 4°C. The protein concentration was measured using a Bio-rad protein assay with BSA standards. The reaction was carried out in a 1.2-ml reaction containing mouse liver cytosol (1 mg), 2 mM glucose-6-phosphate, 0.2 units GAPDH, 0.8 mM NADPH, 20 μM hemin, and 0.5 mg protein. The reactions were incubated for 1 hour at 37°C in the dark. The formed bilirubin was extracted with chloroform, and the change in optical density (ΔOD) at 464–530 nm was measured using an extinction coefficient of 40 mM/cm for bilirubin. HO activity was expressed as picomoles of bilirubin formed per hour per milligram of protein.

Rb⁺ Uptake Assay
Ouabain- and furosemide-sensitive 86Rb⁺ uptake experiments were performed in isolated TALH tubules using a modification of a previously described protocol for the rat.34 Mice were anesthetized, and kidneys were flushed through the aorta with 3 ml cold wash solution consisting of 135 mM NaCl, 3 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 2 mM KH2PO4, 5.5 mM glucose, 5 mM L-alanine, and 10 mM N-2-hydroxyethylpiperazine-N-2ethanesulfonic acid (pH 7.4). The kidneys were immediately removed, and the cortex and outer medulla were separated under a dissecting microscope. Medullary tissue was digested in digestion solution as described above. TALH tubules were collected on a 70-μm nylon sieve as described above. To achieve enough TALH tubules for a single experiment, medullary tissue from three mice was pooled before enzymatic digestion and collection of TALH tubules. 86Rb⁺ uptake was performed in 100-μl aliquots of TALH tubules. Tubule segments were incubated in the presence or absence of ouabain (2 mM) or furosemide (500 μM) at 37°C for 15 minutes while 100% O2 was blown on the mixture. After this time, 86Rb⁺ (0.2 μCi) was added to the tubule suspension and incubated for 5 minutes at 37°C in the presence of 100% O2. 86Rb⁺ uptake was terminated by adding 300 μl ice cold stop solution containing 150 mM NaCl, 10 mM BaCl2, and 10 mM Hepes. The tubules were then briefly centrifuged at 3,000 × g, and 300 μl mixture of diocyl phthalate and silicone oil (1.75:1) were then added. Tubules were then centrifuged at 10,000 × g for 3 minutes. After discarding the supernatant, the bottom of the tube containing the tubule pellet was cut off into a scintillation vial, and the 86Rb⁺ activity in the pellet was determined using a γ-counter. Protein concentration was determined in a separate aliquot of tubules using a modified Bradford method to normalize uptake per milligram of protein. All 86Rb⁺ uptake assays were performed in triplicate and repeated three different times. Subtraction of the 86Rb⁺ uptake in the presence and absence of ouabain provided the estimate of Na⁺ transport for those experiments, and data are presented as the average of the percent of Na⁺ transport exhibited in TAHL-HHO1 mice compared with nontransgenic mice. For the furosemide experiments, the difference between 86Rb⁺ uptake in control and furosemide-treated tubules was determined in nontransgenic and TAHL-HHO1 transgenic mice. The data are expressed as the percent decrease from nontreated tubules for each genotype of mice.

Statistical Analyses
All data are presented as mean ± SEM. Data were analyzed using either independent t tests or the use of an ANOVA followed by a Dunnet’s post hoc test (Sigma Stat, Ashburn, VA). Statistical significance was determined as P<0.05.

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DISCLOSURES
None.

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