Influence of dietary NaCl on l-arginine transport in the renal medulla

Tewabech Zewde, Feng Wu, and David L. Mattson
Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

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NITRIC OXIDE (NO) plays an important role as a paracrine and/or autocrine regulator of renal tubular and vascular function (9, 17). Of particular interest has been the role of NO in the control of renal medullary function. Selective inhibition of NO synthase (NOS) in the renal medulla of conscious rats leads to a selective decrease in renal medullary blood flow, the retention of sodium and water, and the development of hypertension (12). Furthermore, selective stimulation of NOS in the renal medulla by infusion of the NOS substrate l-arginine prevented the development of sodium-sensitive hypertension in Dahl salt-sensitive (Dahl S) rats (13). These findings support the hypothesis that NO is an important regulator of renal medullary and/or vascular function.

Several lines of evidence indicate that the endogenous production of NO by NOS is dependent on extracellular l-arginine and cellular l-arginine transport. In vivo micropuncture studies demonstrated that the cellular uptake of l-arginine by the y+ transporter in the macula densa can modulate glomerular capillary pressure (21). Moreover, experiments in isolated, perfused tubular segments indicate that chloride absorption in the thick ascending loop of Henle is inhibited by NO and is dependent on the extracellular concentration of l-arginine (16). Experiments from our laboratory demonstrated that NO production in isolated inner medullary collecting duct (IMCD) is dependent on uptake of extracellular l-arginine (22) and that chronic blockade of l-arginine uptake in the renal medulla with an antisense oligonucleotide against cationic amino acid transporter 1 (CAT1) or infusion of the cationic amino acids L-ornithine and L-lysine leads to a sustained decrease in NO in the renal medulla and the development of systemic hypertension (8). These data indicate that the cellular uptake of l-arginine may indeed be an important regulator of NO production in this part of the kidney.

Experimental evidence indicates that the IMCD is enriched in NOS enzymatic activity (23) yet does not appear to possess the enzymatic machinery required to produce l-arginine (5, 10). Because l-arginine uptake in this tubular segment appears to be mediated by CAT1 (22), the regulation of cellular l-arginine uptake in this segment may be an important site in the regulation of NO production in the kidney with consequential effects of fluid and electrolyte homeostasis and blood pressure regulation. It is not yet known, however, if the activity of the l-arginine transporters in the inner medulla is modulated by physiological stimuli. The current study was therefore designed to determine if dietary NaCl intake affects l-arginine transport in a freshly isolated suspension of IMCD and on the level of CAT1 immunoreactive protein in the renal inner medulla of Sprague-Dawley rats. Further experiments were then performed to determine the influence of blockade of l-arginine uptake in the renal medulla on arterial blood pressure regulation of conscious rats under conditions of low and high NaCl intake.

MATERIALS AND METHODS

Experiments were performed on male Sprague-Dawley rats (250–300 g) obtained from Harlan Laboratories (Madison, WI). The rats were housed in the Animal Resource Center at the Medical College of Wisconsin with rat chow and tap water provided ad libitum. All animal procedures were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Preparation of IMCD cells. Rats were maintained on low (0.4% NaCl, n = 6)-, normal (1.0% NaCl, n = 6), or high (4.0% NaCl, n = 6)-salt rat chow (AIN-76A; Dyets, Bethlehem, PA) for 2 wk before the study. The IMCD cells were freshly prepared as previously described (6, 19, 22) with minor modifications. Rats were euthanized with an overdose of pentobarbital sodium, and the kidneys were

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rapidly removed and hemisected. The renal papilla was excised and immediately placed in ice-cold HEPES buffer solution (in mmol/l): 135 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgSO₄, 1 KH₂PO₄, 5.5 glucose, and 10 HEPES (pH 7.4). The papilla was stripped into small pieces with dissection forceps along its longitudinal axis in a Petri dish filled with ice-cold HEPES buffer under a LEICA MZ32 stereomicroscope at 4°C and transferred into 10 ml of HEPES buffer solution containing 2 mg/ml collagenase (Worthington Biochemical, Freehold, NJ) in a glass flask. The tissue was incubated at 37°C for 60 min and continuously bubbled with 100% oxygen. After the incubation, the cell suspension was aspirated through a Pasteur pipette several times until there were no visible cell clumps. The cells were mixed with DNAase (20 μU/ml) to prevent clumping and centrifuged at 180 g for 2 min. The supernatant was discarded, the pellet resuspended, and the procedure repeated. The IMCD cells were washed once with HEPES buffer (pH 7.4). The protein concentration of the cell suspension was determined using a Coomassie protein assay kit (Pierce, Rockford, IL) with albumin as a standard.

L-Arginine transport assay. Transport assays were performed using a 96-well Millipore multiwell screen assay system with 1.2-μm nylon filters. A 25-μg aliquot of total protein of IMCD cells was incubated with L-[3H]arginine over a range of 10⁻⁴-500 μmol/l in a final incubation volume of 50 μl HEPES buffer (pH 7.4). After incubating at 37°C for 1 min, uptake was terminated by addition of 0.1 ml ice-cold HEPES buffer containing 25 mmol/l cold L-arginine. The mixture was rapidly aspirated and passed over the 1.2-μm pore size filter under low-pressure vacuum filtration. The filters were immediately washed four times with 200 μl ice-cold HEPES buffer/wash containing 25 mmol/l cold L-arginine. The filters were then dried and dissolved in 10 ml of Ecoscint A (National Diagnostics, Atlanta, GA), and the trapped radioactivity was determined in a liquid scintillation counter. In all experiments, nonspecific uptake (at 0°C) measured in parallel incubations was subtracted from the total uptake (at 37°C) to obtain specific uptake.

Western blotting protocol. Rats were maintained on low (n = 4)- or high (n = 4)-sodium rat chow for 2 wk before the study. The animals were euthanized with an overdose of intraperitoneal pentobarbital sodium, and pieces of the renal inner medulla were rapidly removed and frozen on dry ice. The tissue was stored at −80°C until extraction. In the extraction procedure, pieces of whole tissue were homogenized using a Potter-Elvehjem tissue grinder in a solution containing (in mmol/l) 250 sucrose, 1 EDTA, 0.1 phenylmethylsulfonyl fluoride, and 5 potassium phosphate, pH 7.7. Large tissue debris and nuclear fragments were removed by a low-speed centrifugation step (10,000 g, 4°C, 15 min). The protein concentration of the tissue homogenate was determined by a Coomassie protein assay (Pierce, Rockford, IL) with albumin as a standard.

Protein samples were electrophoretically separated using a discontinuous system consisting of a 7.5% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel. High-range molecular weight markers (~40–200 KDa) were loaded into one lane as a size standard. Equivalent amounts of total protein from the same tissue from rats on a low- and high-salt diet were added to adjacent lanes and the samples were run at 200 V for 45–60 min on an 8 × 10 cm electrophoresis cell (Bio-Rad, Hercules, CA). After separation, the proteins were electrophoretically transferred to a nitrocellulose membrane at 100 V for 1 h. These membranes were washed in TRIS-buffered saline (TBS), blocked with 5% nonfat dried milk in TBS (NFM/TBS) for 2 h, and incubated with a 1:1,000 dilution of rabbit antisera raised against CAT1 in 2% NFM/TBS for 2 h at room temperature. As previously described (8), rabbit antisera was raised against a synthetic peptide corresponding to amino acids 208–226 of rat CAT1 (QLTNKSSLPCGNNDTVK, Alpha Diagnostic, TX). The membranes were then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG in 2% NFM/TBS for 2 h. The bound antibody was detected by chemiluminescence (ECL, Amersham, Arlington Heights, IL) on X-ray film. A monoclonal mouse antibody raised against the structural protein β-actin was used as a loading control. Membranes were stripped between incubations with the different antibodies in a Tris-buffered solution containing 2% SDS and 100 mmol/l β-mercaptoethanol at 50°C.

Surgical preparation and studies in conscious rats. All surgical procedures were similar to those we have previously described. Rats were initially anesthetized with a mixture of ketamine (50 mg/kg im) and acepromazine (5 mg/kg im), and the right kidney was removed. After 10 days of recovery, a polyvinyl catheter was placed in the femoral artery for the measurement of arterial pressure. In addition, the left kidney was exposed by a flank incision and a renal interstitial catheter was inserted into the inner stripe of the outer medulla (~5 mm depth) and secured in place with cyanoacrylate adhesive. The catheters were tunneled subcutaneously, exteriorized at the back of the neck in a lightweight tethering spring, and the interstitial catheter was brought through a spring and swivel for continuous infusion. The animals were kept warm during and after surgery on a specially designed warming table. Buprenex (0.1 mg/kg sc) and penicillin (40,000 U im) were administered postoperatively to control pain and infection, respectively.

After surgical instrumentation, each rat was housed individually in a specially designed stainless steel cage and received a continuous medullary interstitial infusion of saline (0.5 ml/h) while being maintained on a low (0.4%) or high (4.0%) NaCl diet with tap water ad libitum. After 5–10 days of recovery, daily mean arterial pressure (MAP) was recorded at the same time (9:00 am-12:00 pm) for 3 control days followed by 5 days when L-ornithine (5 μmol/kg·min⁻¹), L-lysine (5 μmol/kg·min⁻¹), or L-arginine (5 μmol/kg·min⁻¹) plus L-ornithine (5 μmol/kg·min⁻¹) were added to the interstitial infuse. After 5-day infusion of the amino acids, the infusion was switched to saline for 3 subsequent postcontrol days.

Statistical methods. Data are presented as means ± SE. The significance of differences in L-arginine transport in IMCD obtained from animals on different diets was evaluated with a two-way ANOVA with a Tukey post hoc test. The differences in Western blot densitometry were evaluated using an unpaired t-test. Comparison of MAP between groups on different diets was made with a two-way ANOVA and a Tukey test. A confidence level of P < 0.05 was considered significant.

RESULTS

Effect of low, normal, or high dietary NaCl on L-arginine uptake in IMCD cells. The uptake of L-[3H]arginine into an IMCD cell suspension isolated from rats fed with low (0.4% NaCl, n = 6), normal (1% NaCl, n = 6), or high (4% NaCl, n = 6)-sodium chow is shown in Fig. 1. In rats exposed to a low-sodium (0.4% NaCl) diet, the uptake of L-arginine by IMCD at 500 μmol/l was significantly greater (663 ± 100 pmol·mg⁻¹·min⁻¹, n = 6) compared with rats on a normal (1% NaCl, 519 ± 78 pmol·mg⁻¹·min⁻¹, n = 6) or a high-sodium diet (4.0% NaCl, 302 ± 27 pmol·mg⁻¹·min⁻¹, n = 6). Significant differences were detected between the groups at substrate concentrations of 100 and 250 μmol/l as well. The Eadie-Hofstee transformation of these data indicated a single carrier-mediated system in each group of IMCD cells with calculated Km = 150 μmol/l, 120 μmol/l, and 113 μmol/l on high-, normal, and low-sodium intakes, respectively.

CAT1 immunoreactive protein in the inner medulla. Previous work from our lab has indicated that CAT1 is the y⁺ transporter predominantly expressed in the rat renal medulla (8, 22). A representative Western blot demonstrating the differences in CAT1 immunoreactive protein in the inner medulla of rats maintained on low (0.4% NaCl)- or high (4% NaCl)-salt diets is shown in Fig. 2. As we previously described, this
antibody recognizes a protein of ~80 kDa that is present in the kidney and brain but absent in the liver. Densitometric analysis indicated significantly less CAT1 immunoreactive protein in the inner medulla of rats maintained on the high-sodium diet (4,516 ± 240 densitometric units, n = 4) compared with rats maintained on a low-sodium diet (14,328 ± 302 densitometric units, n = 4). The intensity of the β-actin bands was not different between the groups, indicating equal protein loading between lanes.

**Influence of blockade of L-arginine uptake in the renal medulla on blood pressure of conscious rats maintained on a low- or high-NaCl diet.** Mean arterial blood pressure was significantly increased after 5 days of renal medullary interstitial infusion of L-lysine or L-ornithine in rats on either the low (0.4% NaCl)- or high (4% NaCl)-salt diet. After 5 days of infusion of L-lysine, MAP increased from 120 ± 5 to 141 ± 2 mmHg in rats (n = 5) maintained on a low-sodium diet and from 123 ± 2 to 141 ± 2 mmHg in rats (n = 5) maintained on high-sodium diet (Fig. 3). Similar effects were observed after 5 days of medullary interstitial infusion of L-ornithine (Fig. 4). In the low-NaCl groups, MAP increased from 120 ± 2 to 138 ± 2 mmHg (n = 5), and in the high-NaCl group, MAP increased from 123 ± 2 to 145 ± 2 mmHg (n = 5). There was no significant difference in the increase in MAP reached during infusion of either L-lysine or L-ornithine between the rats administered the low- or high-sodium diet. To control for potential nonspecific effects of infusion of the other cationic amino acids, an additional group of rats was prepared and infused with equal amounts of L-arginine and L-ornithine. As shown in Fig. 5, coinfusion of L-arginine with L-ornithine into the renal medullary interstitial space prevented the increase in arterial blood pressure associated with L-ornithine infusion to rats maintained on the high-sodium diet.

**DISCUSSION**

We previously demonstrated that L-arginine uptake in IMCD is predominately mediated by a high-affinity y+ transporter encoded by CAT1 (22). The present work demonstrated that cellular L-arginine uptake in the IMCD from rats maintained on a high-sodium diet is significantly inhibited compared with that of rats on a normal or a low-sodium diet. In agreement with that finding, immunoblotting studies demonstrated that CAT1 immunoreactive protein was significantly decreased in the inner medulla of rats maintained on a high NaCl intake, indicating that CAT1 protein in the IMCD or other cell types in the renal medulla was reduced when the rats were placed on high salt. Despite the marked changes observed in L-arginine uptake and CAT1 immunoreactive protein during changes in sodium intake, no difference was detected in the long-term blood pressure response during renal medullary interstitial infusion of L-ornithine or L-lysine. These functional

![Fig. 1. Kinetics of L-arginine transport into inner medullary collecting duct (IMCD) cells from rats fed with low (0.4% NaCl)-, normal (1% NaCl), and high (4% NaCl)-salt diets. Uptake was measured for 1 min using increasing concentrations (10–500 μmol/L) of L-[3H]arginine in a final reaction volume of 50 μL. Results are means ± SE of 6 separate experiments. *P < 0.05 from high NaCl group at equivalent L-arginine concentration.](image1)

![Fig. 2. Representative Western blot of cationic amino acid transporter 1 (CAT1) protein in total tissue protein isolated from the inner medulla of Sprague-Dawley rats fed with low (0.4% NaCl; LS)- or high (4% NaCl; HS)-salt diets. β-actin served as an internal control demonstrating the equal loading of protein in each lane.](image2)

![Fig. 3. Daily mean arterial pressure in Sprague-Dawley rats maintained on low (0.4% NaCl)- or high (4.0% NaCl)-salt diets during the control period (C1–C3), during the experimental period in which L-lysine (E1–E5, 5 μmol/kg−1·min−1, n = 5) was infused into the renal medullary interstitial space, and during the postcontrol (P1–P3) period. *P < 0.05 from C3.](image3)

![Fig. 4. Daily mean arterial pressure in Sprague-Dawley rats maintained on low (0.4% NaCl)- or high (4.0% NaCl)-salt diets during the control period (C1–C3), during the experimental period in which L-lysine (E1–E5, 5 μmol/kg−1·min−1, n = 5) was infused into the renal medullary interstitial space, and during the postcontrol (P1–P3) period. *P < 0.05 from C3.](image4)
studies therefore indicate that the observed alterations in l-arginine uptake do not have a functional role in the regulation of arterial blood pressure in normal animals during changes in sodium intake.

The potential importance of l-arginine uptake in the regulation of NO formation with a subsequent influence on fluid and electrolyte homeostasis and blood pressure regulation is emphasized by the results of several different studies that indicate that l-arginine uptake is important in NO formation in a number of different cell types. The cellular uptake of l-arginine by the y+ transporter in the macula densa can modulate glomerular capillary pressure (21), leads to the inhibition of chloride reabsorption by the isolated perfused thick ascending limb (16), and is important for NO production in isolated IMCD (22). Moreover, chronic oral or intravenous l-arginine supplementation can prevent sodium-induced hypertension in the Dahl S rats (2, 3, 7). Consistent with the prevention of hypertension in conscious Dahl S rats, l-arginine administration led to a normalization of the pressure-natriuresis relationship and increased transmission of perfusion pressure to the kidney in anesthetized Dahl S rats (14, 15). Interestingly, intravenous l-arginine infusion has been demonstrated to increase renal medullary interstitial NO levels (24), whereas selective infusion of l-arginine into the renal medulla of Dahl S rats, in a dose that had no effect when delivered intravenously, prevented the development of hypertension in this model (13).

The present results are consistent with recent studies from our lab that demonstrated that chronic blockade of l-arginine uptake in the renal medulla with an antisense oligonucleotide against CAT1 or the cationic amino acids l-ornithine and l-lysine leads to a sustained decrease in NO in the renal medulla and the development of systemic hypertension (8). Cellular l-arginine uptake therefore appears capable of playing a key role in the regulation of NO production with consequential effects on renal sodium handling, the maintenance of extracellular fluid volume, and the regulation of arterial blood pressure. The effects of infusion of the amino acids into the renal medulla in the present study and our previous data that demonstrate that renal medullary infusion of these amino acids is associated with selective alterations in NO in the renal medulla indicate that the mechanism of hypertension is associated with modulation of l-arginine uptake and NO production from cell types in this region of the kidney. We cannot determine from the present studies, however, which cell types in the medulla or the deep renal cortex are responsible for the functional effects on arterial blood pressure. Moreover, it is possible that the interruption of l-arginine uptake in these cell types leads to alterations in arginase activity, alterations in enzymes of the urea cycle or even changes in arginine excretion that could potentially influence arterial blood pressure by NO-independent mechanisms.

Several lines of evidence have indicated that dietary salt loading leads to an increase in NO production as demonstrated by increased excretion of nitrite and nitrate and cGMP (1, 18, 20). An increase in NOS immunoreactive protein in the rat renal medulla has also been observed during dietary salt loading (11). Furthermore, blockade of NOS by l-NAME increased renal vascular resistance and MAP to a greater extent in rats during adaptation to a high-salt diet compared with rats on a low-salt diet (11). It has been suggested that during low salt intake, decreased availability of l-arginine downregulates NO production and blunts the vasoconstrictor response to NOS inhibition (4). The present studies were therefore performed to determine if sodium intake also altered l-arginine uptake in the renal medulla and the functional responses that result as a consequence of long-term blockade of arginine uptake in the medulla. In the current study, blockade of l-arginine uptake in the renal medulla led to a sustained increase in MAP in both high and low salt intake rats. Moreover, there was no significant difference in the increase in MAP observed in the rats maintained on high and low sodium intake. This was observed despite decreased l-arginine uptake and CAT1 immunoreactive protein in the renal medulla of rats maintained on a high-sodium diet. The apparent downregulation of arginine uptake mechanisms in the renal medulla does not therefore appear to have a sustained adverse effect on fluid and electrolyte homeostasis and blood pressure regulation in the normal SD rat.

Despite data indicating the potential importance of l-arginine uptake in the regulation of renal medullary function, the significance of the observed changes in l-arginine uptake during alteration in dietary sodium intake is not clear. It has been observed that NOS protein and enzymatic activity in the renal medulla, particularly in the IMCD, is much higher than that in the cortex and outer medulla (11, 13, 23). Furthermore, dietary sodium loading led to a significant increase of the NOS immunoreactive protein in the renal inner medulla (11). However, the results from the current study suggest that increased NOS activity of IMCD cells of salt-loaded rats in the l-arginine/NO pathway may encounter a reduction in CAT1 protein and reduced l-arginine transport activity in the same region of the kidney. Although the reduction in l-arginine uptake capacity did not lead to alterations in arterial blood pressure in the normal rats during changes in sodium intake, the reduction in the cellular l-arginine uptake capacity during an elevation in sodium intake may be an important factor in rats or other animals in which blood pressure is sensitive to sodium intake. Further experiments remain to be performed to determine if there is indeed a change in cellular l-arginine uptake in vivo.

In conclusion, results of the present study demonstrate that long-term salt loading leads to a significant reduction in


1-arginine uptake in isolated IMCD and a reduction in CAT1 protein in the renal inner medulla of Sprague-Dawley rats. The decrease in 1-arginine uptake capacity, however, does not lead to alterations in the blood pressure response to 1-arginine uptake inhibition in the renal medulla. Further investigation is required to discern the mechanism(s) leading to these changes and the functional importance of alterations in 1-arginine uptake in the renal medulla.

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