Cationic Amino Acid Transport in the Renal Medulla and Blood Pressure Regulation

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Abstract—Previous studies have indicated that NO synthesis in isolated inner medullary collecting duct cells is reduced by cationic amino acids that compete with L-arginine for cellular uptake. In the present study, we investigated the effects of chronic renal medullary infusion of cationic amino acids on renal NO concentration and mean arterial pressure (MAP) in Sprague-Dawley rats. Renal medullary infusion of L-ornithine (50 μg/kg per min) or L-lysine (50 μg/kg per min) markedly decreased NO in the medulla (vehicle, 124±11 nmol/L; L-ornithine, 45±4 nmol/L; L-lysine, 42±6 nmol/L) and increased MAP (vehicle, 111±7 mm Hg; L-ornithine, 143±6 mm Hg; L-lysine, 148±3 mm Hg) after 5 days of infusion. In contrast, intravenous infusion of the same dose of L-ornithine or L-lysine for 5 days increased plasma concentration to levels similar to those observed with intramedullary infusion but did not change NO in the medulla or alter MAP. Furthermore, the NO-suppressing and hypertensive effects of medullary interstitial infusion of L-ornithine (50 μg/kg per min) were attenuated by simultaneous infusion of L-arginine (500 μg/kg per min; NO, 97±10 nmol/L; MAP, 124±3 mm Hg). A 5-day infusion of an antisense oligonucleotide against CAT-1 (18-mer, 8.3 nmol/h) significantly decreased CAT-1 protein in the medulla, decreased NO in the medulla (scrambled oligo, 124±10 nmol/L; antisense oligo, 67±11 nmol/L), and increased MAP (scrambled oligo, 113±2 mm Hg; antisense oligo, 130±2 mm Hg). These results suggest that uptake of L-arginine by cationic amino acid transport systems in the renal medulla plays an important role in the regulation of medullary NO and MAP in rats. (Hypertension. 2002;39:287-292.)

Key Words: arterial pressure □ amino acid □ kidney □ nitric oxide □ renal blood flow

Nitric oxide plays an important role in regulation of vascular tone, renal sodium handling, and systemic blood pressure. The inhibition of NO synthase (NOS) with inactive L-arginine analogs indicates that the availability of L-arginine, the substrate for NOS, is important for the generation of NO. L-Arginine is supplied by intracellular synthesis and/or uptake from the extracellular space. In the cytoplasm, L-arginine is converted from L-citrulline by argininosuccinate synthase or lyase, or is produced from protein breakdown. Extracellular L-arginine may also provide NOS substrate by transport across plasma membranes via amino acid transport systems denoted by y+ or y+, B0+, B0,+ or γ+L, although not all transporters are expressed in every cell type.

Of the different cationic amino acid transport systems, system y+ is considered to be responsible for the majority of L-arginine transport, is widely distributed in a variety of tissues, and is encoded by the cationic amino acid transporter (CAT) genes. To date, at least 4 CAT isoforms have been identified in mammals, ie, CAT-1, CAT-2, CAT-2a, and CAT-3. In the rat kidney, CAT-1, CAT-2, and CAT-3 mRNA have been detected by reverse transcription–polymerase chain reaction, but CAT-1 appears to predominate, especially in structures of the renal medulla. In addition, our previous studies have indicated that NO synthesis in isolated inner medullary collecting duct cells is substantially reduced by the cationic amino acids L-ornithine and L-lysine, which compete with L-arginine for cellular uptake. Studies from our laboratory have demonstrated that NO in the renal medulla is important in the regulation of mean arterial pressure (MAP). To explore whether chronic inhibition of L-arginine uptake in the renal medulla reduces NO concentration and elevates MAP, we examined the influence of renal medullary interstitial infusion of cationic amino acids and an antisense oligonucleotide for CAT-1, the predominant CAT isoform in the renal medulla, on regional NO concentration and MAP in conscious rats.

Methods

Animals and Surgical Preparation

Male Sprague-Dawley rats (300 to 350 g) were purchased from Harlan Sprague Dawley (Madison, Wis). Normal rat chow and water were available ad libitum. Animal procedures were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Rats were anesthetized with ketamine (50 mg/kg IM) and acepromazine (2 mg/kg IM), and the right kidney was removed. Uninephrectomy was necessary to determine the influence of renal medullary infusion on blood pressure because interstitial catheters are only placed in 1 kidney. Approximately 10 days later, catheters...
were implanted in the femoral artery, femoral vein, or renal medullary interstitium as described.6

Analytical Methods
Plasma amino acid concentration was determined by high-performance liquid chromatography separation and fluorometric quantification as described.6 NO was measured in the renal interstitium by microdialysis-oxyhemoglobin trapping as described.9

Immunoblot Analysis
Rabbit antiserum was raised against a synthetic peptide (amino acids 208 to 226 of rat CAT-1, QLTENKSGCNGNTDVNK; Alpha Diagnostics). Tissue homogenization and Western blotting were performed as described.9

Semiquantification of Intracellular NO by DAF-2 Fluorescence
Isolated renal inner medullary cells were prepared as described4 from kidneys infused with oligonucleotides. The cells (100 μg of protein) were incubated 1 hour at 37 °C in HEPES buffer (100 μL) containing 10 μmol/L 4,5-diaminofluorescein (DAF-2) diacetate with 0.2% DMSO in a 96-well polystyrene microtiter plate (96-Well Plate, Maxisorp, VWR). In some groups, l-arginine (1 mmol/L), N3-nitro-l-arginine methyl ester (L-NAME, 1 mmol/L), or authentic NO (1 μmol/L) was added to the cells. In 1 group, cell membranes were disrupted by sonication (60 Sonic Dismembrator, Fisher Scientific) before the incubation. The intensity of DAF-2 fluorescence was evaluated using a microplate fluorescence reader (FL600, Bio-Tek; excitation, 475 to 495 nm; emission, 515 to 535 nm); background fluorescence was not subtracted in this experiment.

Protocols
After instrumentation, saline infusion (0.5 mL/h) was begun. After 5 recovery days, daily MAP measurements began. After 3 control days, rats were infused with l-ornithine (50 μg/kg per min) or l-lysine (50 μg/kg per min) through the intramedullary catheter for 5 days. The influence of intramedullary co-infusion of excessive l-arginine (500 μg/kg per min) with l-ornithine (50 μg/kg per min) was also examined. To control for recirculation of these amino acids, the influence of intravenous infusion of the same dose of l-ornithine or l-lysine was examined. To determine the effects of l-arginine uptake inhibition with a different method, we examined the effect of intramedullary infusion of a phosphorothioated antisense oligodeoxynucleotide for CAT-1 (18-mer, 5'-GGTTTTTCGGACCCCATCGG-3'; 8.3 nmol/h) on MAP. A phosphorothioated-scrambled oligodeoxynucleotide (18-mer, 5'-TAGTCGACGTCCGTACG-3'; 8.3 nmol/h) was used as the control. After infusion of the antisense and scrambled oligonucleotides, CAT-1 protein was studied by Western blotting. In separate experiments, rats were set up for the 5-day infusions and anesthetized for acute measurement of NO in the renal cortex and medulla by in vivo microdialysis-oxyhemoglobin trapping or for the DAF-2 study in freshly isolated cells.

Statistics
Data are expressed as mean±SEM. Statistical comparisons were made with a 1-way ANOVA for repeated measures and a Student-Neumann-Keuls test. Differences with P<0.05 were considered significant.

Results
Effect of Chronic Renal Medullary Infusion of l-Ornithine and l-Lysine on MAP
As shown in Figure 1 (top), renal medullary infusion of l-ornithine (50 μg/kg per min) or l-lysine (50 μg/kg per min) for 5 days markedly elevated MAP (l-ornithine [n=6], 143±6 mm Hg; l-lysine [n=6], 148±3 mm Hg). In contrast, there was no alteration in arterial pressure in control rats in which saline was infused interstitially (vehicle [n=6], 111±7 mm Hg). Furthermore, the hypertensive effect of medullary interstitial infusion of l-ornithine (50 μg/kg per min) was attenuated by simultaneous infusion of l-arginine (500 μg/kg per min; n=6, 124±3 mm Hg). The plasma concentration of l-ornithine, l-lysine, and l-arginine averaged 55.0±3.5, 367.2±19.8, and 112.5±8.4 μmol/L, respectively, in a group of control rats (n=6). After the 5-day renal medullary interstitial infusion of the amino acids to the different groups, the circulating concentration of l-ornithine was significantly increased to 104.1±6.9 μmol/L (n=6); l-lysine was significantly increased to 415.5±20.4 μmol/L (n=6); and l-arginine was significantly increased to 228.2±9.5 μmol/L (n=6).

Effect of Chronic Intravenous Infusion of l-Ornithine and l-Lysine on MAP
As shown in Figure 1 (bottom), intravenous infusion of the same dose of l-ornithine (50 μg/kg per min) or l-lysine (50 μg/kg per min) that led to an increase in MAP when infused into the renal medulla for 5 days did not alter MAP in
conscious heminephrectomized Sprague-Dawley rats (L-ornithine [n=6], 111±7 mm Hg; L-lysine [n=6], 112±5 mm Hg). Similarly, control rats in which saline was infused intravenously had no significant change in MAP (vehicle [n=6], 107±5 mm Hg). Interestingly, the circulating levels of both L-ornithine and L-lysine after a 5-day intravenous infusion were significantly elevated from the control rats (118.1±15.8 and 429.1±22.4 μmol/L [both, n=6], respectively) but not different from that observed with renal medullary interstitial infusion.

Effect of Chronic Renal Medullary Infusion of Cationic Amino Acids and Oligonucleotides on NO Concentration and CAT-1 Expression in the Renal Cortex and Medulla

As shown in Figure 2 (top), NO concentration in the renal medulla into which L-ornithine (50 μg/kg per min) or L-lysine (50 μg/kg per min) was infused for 5 days was markedly lower than that in which saline was infused (vehicle, 124±11 nmol/L; L-ornithine, 45±4 nmol/L; L-lysine, 42±6 nmol/L). However, simultaneous infusion of an excessive dose of L-arginine (500 μg/kg per min) with L-ornithine (50 μg/kg per min) into the renal medulla significantly attenuated the decrease in NO in the medulla by L-ornithine (97±10 nmol/L). As shown in Figure 2 (bottom), the NO concentration in the renal medulla of rats in which L-ornithine or L-lysine was infused intravenously for 5 days was not significantly different from that in which saline was infused (vehicle, 120±11 nmol/L; L-ornithine, 108±12 nmol/L; L-lysine, 99±10 nmol/L). No significant alteration in NO was observed in the renal cortex was observed by intramedullary infusion of L-ornithine, L-lysine, or L-ornithine and L-arginine (vehicle, 87±8 nmol/L; L-ornithine, 78±9 nmol/L; L-lysine, 74±5 nmol/L; L-ornithine and L-arginine, 88±10 nmol/L) or by intravenous infusion of L-ornithine or L-lysine (vehicle, 81±11 nmol/L; L-ornithine, 75±7 nmol/L; L-lysine, 72±8 nmol/L). The amount of CAT-1 protein in the renal medulla of heminephrectomized Sprague-Dawley rats after 5-day infusion of L-ornithine was also examined (Figure 3). Compared with that in control rats, immunoreactive CAT1 protein was decreased by 59±4% in the medulla of rats infused with L-ornithine. β-actin was not different between groups.

Effect of Chronic Renal Medullary infusion of Antisense Oligonucleotides for CAT-1 on MAP, CAT-1 Protein, and NO Concentration in the Renal Medulla

A 5-day infusion of the antisense oligonucleotide for CAT-1 (18-mer, 8.3 nmol/h) significantly increased MAP (Figure 4) compared with that of a group that received a scrambled oligonucleotide (scrambled oligo [n=6], 113±2 mm Hg; antisense oligo [n=6], 130±2 mm Hg). As shown in Figure 5 (top), the amount of medullary CAT-1 protein was markedly suppressed by intramedullary infusion of an antisense oligonucleotide for CAT-1. Densitometric analysis indicated that CAT-1 protein was decreased by 76±7% in the antisense-treated group. As shown in Figure 5 (bottom), the NO concentration in the renal medulla of rats in which the antisense oligonucleotide for CAT-1 was infused for 5 days was significantly lower than that of rats into which scrambled oligonucleotide was infused (scrambled oligo, 124±10 nmol/L; antisense oligo, 67±11 nmol/L). In contrast, no significant alteration in NO was observed in the renal medulla.
cortex during intramedullary infusion of the antisense or scrambled oligonucleotide (scrambled oligo, 79±6 nmol/L; antisense oligo, 78±8 nmol/L).

Effect of Chronic Renal Medullary Infusion of Antisense Oligonucleotides for CAT-1 on Intracellular NO in Freshly Isolated Renal Medullary Cells

As shown in Figure 6, the DAF-2 fluorescence without L-arginine or in the presence of 1 mmol/L L-NAME was the same between cells from rats infused with the antisense (n=4) and scrambled (n=4) oligonucleotides. In cells incubated with 1 mmol/L L-arginine, however, the DAF-2 signal was significantly lower in cells isolated from the antisense oligonucleotide–treated rats compared with the cells from rats treated with the scrambled oligonucleotide, indicating that less NO was generated in the cells from the antisense-treated rats. Predisruption of the cell membrane in addition to the L-arginine abolished the difference between the 2 groups, indicating that NOS activity was not affected by the antisense treatment. The addition of 1 μmol/L authentic NO solution at the beginning of the incubation caused a comparable elevation of DAF-2 fluorescence in the antisense and scrambled groups, suggesting equal uptake of dye between the 2 groups.

Discussion

Studies from our laboratory have demonstrated that NO in the renal medulla plays an important role in long-term control of MAP in rats. Direct infusion of the NOS inhibitor L-NAME into the renal medulla led to the retention of sodium and the development of hypertension. In contrast to the hypertensive effects of NOS inhibition, chronic supplementation of NOS substrate L-arginine in the renal medulla prevented the decrease in medullary blood flow and the development of hypertension that occurs in Dahl salt-sensitive rats when placed on a high-sodium diet. The present data indicate that blockade of L-arginine uptake in the renal medulla of uninephrectomized Sprague-Dawley rats decreases NO in the medulla and leads to a sustained elevation of MAP.

In cytokine-stimulated macrophages, astrocytes, and vascular smooth muscle cells, the great proportion of NO generated is dependent on the uptake of extracellular L-arginine. Furthermore, it has been demonstrated that NO-mediated effects on tubuloglomerular feedback in the kidney are attenuated by L-lysine and L-homoarginine. It is important to note, however, that NO production in cultured endothelial cells is not significantly suppressed by L-lysine. The decrease in renal medullary NO observed in the present study during interstitial infusion of cationic amino acids may therefore be caused by alterations in L-arginine uptake in nonendothelial cells. Consistent with this concept are our previous studies that suggested that NO production in the inner medullary collecting duct is dependent on cellular L-arginine uptake. In addition to the competitive inhibition of L-arginine uptake by cationic amino acids (cis-inhibition), the cationic amino acids may also facilitate the exodus of intracellular L-arginine from cells (trans-stimulation) and thereby deplete the cells of L-arginine. The mechanism of inhibition cannot be determined from the present studies, although simultaneous intramedullary infusion of an excessive dose of L-arginine with L-ornithine restored NO in the renal medulla and attenuated the increase in MAP. Although infusion of a 10-fold excess of L-arginine attenuated the L-ornithine–induced hypertension, it did not lower MAP in these experiments. This observation is consistent with a previous study in which we observed that infusion of L-arginine alone did not alter blood pressure in normotensive Wistar-Kyoto or Dahl salt-resistant rats, although that same dose of L-arginine prevented the development of hypertension in Dahl salt-sensitive rats. An explanation for the lack of effect of L-arginine is unclear, because we recently observed that acute intramedullary infusion of L-arginine to anesthetized Sprague-Dawley rats leads to an increase in NO in the medulla and an increase in medullary blood flow (M. Kakoki and D.L. Mattson, unpublished observations, 2001).

The major pathways of L-arginine synthesis include arginosuccinate synthase and lyase, which produce L-arginine from L-citrulline, and protein breakdown. It has previously been reported that arginosuccinate synthase and lyase activity in the kidney is isolated primarily to the renal cortex. It is possible that the uptake of exogenous...
L-arginine from the extracellular space is the predominant L-arginine supply in the renal medulla. Because NO concentration\(^6\) and the expression and activity of NOS\(^{18,19}\) is higher in the renal medulla than in the renal cortex, the L-arginine consumption in this part of the kidney may also be greater and increase the dependence on extracellular L-arginine uptake.

A previous study from our laboratory using a radiolabeled calcium antagonist demonstrated that intramedullary infusion concentrates infused compounds in the renal medulla.\(^{20}\) Interestingly, the plasma concentrations of L-ornithine and L-lysine were similarly elevated by intramedullary and intravenous infusion. Despite the similar increase in circulating L-ornithine and L-lysine, only intramedullary infusion of these amino acids decreased medullary NO and elevated MAP, suggesting that renal medullary infusion of L-ornithine and L-lysine did not elevate MAP by escape and recirculation but that locally high concentration of these amino acids decreased NO in the renal medulla to levels that resulted in systemic hypertension.

To confirm the effects of the cationic amino acids, an antisense oligonucleotide for CAT-1 was chronically administered to additional rats. Treatment with the antisense oligonucleotide decreased CAT-1 protein in the medulla, decreased NO in the medulla, decreased NO production in freshly isolated cells exposed to L-arginine, and led to the development of hypertension. These experiments confirm the results obtained with the cationic amino acids and indicate that the CAT-1 transporter has an important role in the regulation of NO formation in the kidney. It is important to note that CAT-1 is widely distributed in a number of tissues and is important in a number of cellular processes. A recent gene knockout study demonstrated that CAT-1–null mutant mice are 25% smaller in body weight than wild-type controls, are anemic, and do not survive past the day of birth.\(^{21}\) In contrast to gene knockout studies, the present experimental approach permits the examination of selective inhibition of CAT-1 in the kidney.

A number of methods were used to confirm that the antisense oligonucleotide blocked CAT-1. First, there was significantly less immunoreactive CAT-1 protein in the medulla of the antisense-treated rats compared with the rats treated with the scrambled oligonucleotide. Second, the NO concentration was significantly less in the medulla of antisense-treated rats compared with scrambled oligonucleotide–treated rats. Third, freshly isolated cells from antisense-treated rats showed less of an increase in NO when incubated with L-arginine in comparison to cells isolated from the scrambled oligonucleotide–treated rats. In addition, in the isolated cell studies, the similar fluorescence induced by authentic NO between the 2 groups suggests little difference in the uptake of the dye between the 2 groups, and the normalization of DAF-2 signal in cells in which the membranes were disrupted indicates that NOS activity was not affected by the antisense treatment.

Despite the similar effects on blood pressure observed after medullary interstitial infusion of the antisense oligonucleotide for CAT-1 and the cationic amino acids, the antisense treatment was quantitatively less effective at lowering NO and elevating systemic arterial blood pressure in comparison to chronic L-ornithine and L-lysine infusion. One possible explanation for this difference is that other cationic transporters may have been upregulated during antisense inhibition of CAT-1. Previous studies have reported that the expression of CAT-2 mRNA and CAT-3 mRNA and protein were compensatorily upregulated in embryonic fibroblast cells derived from CAT-1 knockout mice.\(^{22}\) Moreover, other cationic transport systems, ie, system b\(^\text{0}\)\(^{-}\) and y\(^+\)L, have been shown to exist in the mammalian kidney\(^{23,24}\) and may also contribute to L-arginine uptake in NO-producing cells. A further possibility is that the cationic amino acids have a dual effect on L-arginine: (1) they may competitively inhibit L-arginine uptake by cationic amino acids (cis-inhibition) and (2) the cationic amino acids may also facilitate the exodus of intracellular L-arginine from cells via system y\(^-\) (trans-stimulation) and thereby deplete the cells of L-arginine.\(^{15}\) It is therefore possible that the cationic amino acids not only competitively inhibit the uptake of extracellular L-arginine but may also deprived NO-producing cells of L-arginine present in the intracellular space. An additional observation in this study was the influence of chronic L-ornithine infusion to decrease CAT-1 protein. The mechanism of this effect in the present study is unclear, although it has been demonstrated previously that amino acid starvaton results in an increased CAT-1 mRNA level to support synthesis of additional CAT-1 protein.\(^{23,26}\) In the present study, the delivery of excess L-ornithine may have been sufficient to reduce CAT-1 expression, although this observation remains to be further characterized. The change in CAT-1 expression during chronic L-ornithine or L-lysine infusion may be an additional mechanism, in addition to the competition with L-arginine for cellular uptake, whereby the infusion of cationic amino acids decreased NO in the renal medulla and elevated MAP.

These studies demonstrate that chronic renal medullary infusion of cationic amino acids or an antisense oligonucleotide against CAT-1 leads to decreased NO levels and hypertension in normal rats. These results suggest that uptake of L-arginine by cationic amino acid transport systems in the renal medulla plays an important role in the regulation of medullary NO and MAP.

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