Characterization of L-arginine transporters in rat renal inner medullary collecting duct

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Wu, Feng, Brian Cholewa, and David L. Mattson. Characterization of L-arginine transporters in rat renal inner medullary collecting duct. Am J Physiol Regulatory Integrative Comp Physiol 278: R1506–R1512, 2000.—Previous work from our laboratory has demonstrated that the inner medullary collecting duct (IMCD) expresses a large amount of nitric oxide synthase (NOS) activity. The present study was designed to characterize the transport of NOS substrate, L-arginine, in a suspension of bulk-isolated IMCD cells from the Sprague-Dawley rat kidney. Biochemical transport studies demonstrated an L-arginine transport system in IMCD cells that was saturable and Na+ independent (n = 6). L-Arginine uptake by IMCD cells was inhibited by the cationic amino acids L-lysine, L-homoarginine, and L-ornithine (10 mmol/l each) and unaffected by the neutral amino acids L-leucine, L-serine, and L-glutamine. Both L-ornithine (n = 6) and L-lysine (n = 6) inhibited NOS enzymatic activity in a dose-dependent manner in IMCD cells, supporting the important role of L-arginine transport for NO production by this tubular segment. Furthermore, RT-PCR of microdissected IMCD confirmed the presence of cationic amino acid transporter CAT1 mRNA, whereas CAT2A, CAT2B, and CAT3 were not detected. These results indicate that L-arginine uptake by IMCD cells occurs via system y+", is encoded by CAT1, and may participate in the regulation of NO production in this renal segment.

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distinct proteins encoded by alternatively spliced mRNAs originated from one primary transcript of the CAT2 gene (5, 20, 21). CAT2A has been found in hepatocytes (5), whereas CAT2B was first detected in activated thymocytes and has recently been described in many cell types including brain, heart, lung, and testis (7, 17, 20, 21, 35, 37). A fourth member of the cationic amino acids transport family, CAT3, was recently cloned from rat brain (14).

The present study was designed to characterize L-arginine transport in a suspension of bulk-isolated IMCD cells of the Sprague-Dawley rat kidney by competition-inhibition assay and kinetic studies of L-arginine uptake. Following the biochemical characterization of L-arginine transport, RT-PCR was used to determine which CAT transporter(s) is expressed in the IMCD. A final set of studies was then performed to elucidate the importance of L-arginine transport on NOS enzymatic activity in a suspension of intact, isolated IMCD cells.

**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 normal 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. The preparation of the IMCD cell suspension was performed as previously described (11, 38) with minor modifications. Rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip), and the kidneys were rapidly removed and hemisected. The renal papilla was excised and immediately placed in ice-cold HEPES buffer (pH 7.4), and 25 µg of total protein from IMCD was determined using a Coomassie protein assay kit (Pierce, Rockford, IL) with albumin as a standard.

Transport assays were performed using a 96-well Millipore multiscrreen assay system with 1.2-µm nylon filters. The IMCD cells were washed with HEPES buffer (pH 7.4), and 25 µg of total protein from IMCD cells were added into each reaction tube. For L-[3H]arginine uptake determination, 25 µg of the IMCD cell suspension were incubated with 50 nmol/l L-arginine (with 30,000 cpm L-[3H]arginine, sp act 68 Ci/mmol added as trace); in saturation experiments, the L-arginine concentrations varied over a range of 2–1,000 nmol/l. In some experiments, sodium was replaced in the HEPES buffer by isosmotic replacement with choline. In amino acid competition-inhibition assays, 10 mmol/l of the selected amino acids was added to the uptake medium. After incubating at 37°C for 1 min (except as otherwise indicated), uptake was terminated by addition of 200 µl 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 of 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 Ecosint 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.

NOS activity inhibition assay. An IMCD cell suspension containing 25 µg of protein was incubated with 50 µmol/l L-arginine (with 30,000 cpm L-[3H]arginine, sp act 68 Ci/mmol) in the presence of variable concentrations of L-arginine analogs in 100 µl 20 mmol/l HEPES buffer (pH 7.2) at 37°C for 5 min. The reaction mixture also included 2 mmol/l CaCl2, 1 mmol/l NADPH, 25 µmol/l FAD, 1.25 µg/ml calmodulin, and 10 µmol/l tetrahydrobiopterin. The L-arginine and converted L-citrulline were separated by isocratic reverse-phase HPLC with a Supelco (Bellefonte, PA) model LC-18-DB column (mobile phase 11.5% methanol, 11.5% acetonitrile, 1% tetrahydrofuran, and 0.1 mol/l KH2PO4, pH 5.9), and the amounts of L-[3H]arginine and formed L-[3H]citrulline were quantitated by radiochemical detection (Packard, Tampa, FL). NOS activity was determined as a function of L-citrulline formation.

RNA extraction and RT-PCR of CAT isoforms. Microdissection of IMCD was carried out as described previously (26, 27, 41). To extract total RNA, the microdissected IMCD were washed and placed in individual tubes containing 500 µl TRIzol reagent (Life Technologies), and following vortex mixing, 100 µl of chloroform were added to each tube. The mixtures were centrifuged at 12,000 g for 15 min, and the aqueous phase was removed. The RNA was precipitated with 250 µl isopropanol and washed with 500 µl 75% ethanol. The resultant RNA was allowed to dry at room temperature and dissolved in 8 µl of RNAse-free diethyl pyrocarbonate water.

A first-strand cDNA synthesis kit (Pharmacia Biotech) was used to synthesize cDNA by reverse transcription (RT) from RNA extracted from IMCD. As described by the manufacturer, 8 µl of total RNA were heated at 65°C for 10 min and rapidly chilled on ice, then mixed with 7 µl of the reagents supplied with the kit. The reaction mixture contained 0.2 µg random hexadeoxy nucleotides, 45 mmol/l Tris (pH 8.3), 68 mmol/l KCl, 15 mmol/l diethylthritol, 9 mmol/l MgCl2, 0.08 mg/ml BSA, 1.8 mmol/l dNTPs, and 100 U Moloney murine leukemia virus reverse transcriptase. The reaction mixture was incubated at 37°C for 60 min and then heated to 65°C for 10 min to inactivate the reverse transcriptase and denature cDNA hybrids.

The PCR reactions were performed in a total volume of 50 µl using a PCR Supermix kit (GIBCO BRL) containing 22 mmol/l Tris·HCl (pH 8.4), 55 mmol/l KCl, 1.65 mmol/l MgCl2, 200 µmol/l dNTPs, 3.5 µl RT reaction mixture (1 µl for β-actin), 22 U recombinant Taq DNA polymerase, and 40 pmol of the specific primer pairs for CAT1, CAT2A, CAT2B, and CAT3. The reactions were thermocycled 35 times between 94°C (denaturation) for 1 min, 56°C (annealing) for 1 min, and 72°C (extension) for 1 min. The reactions were extended for an additional 7 min at 72°C after the last cycle was completed. Negative control PCR reactions with a substitution of
nucleotide primers were purchased from Operon Technologies (Alameda, CA). The primer pairs were chosen from the published cDNA sequences of rat CAT1 (1), mouse CAT2A (5), mouse CAT2B (21), and rat CAT3 (14). The primers used for CAT3 were 5'-GCC GGT GAG AAG GTG CAC CAA-3' (sense, bp 30–50) and 5'-GAC GAG GAC ATT GAT ACA GGT G-3' (antisense, bp 679–658); the final PCR product was 650 bp in size. The primers for CAT2A were from the published sequences (9): 5'-CCA CCA TGA TCC CCT GCA GA-3' (sense, bp 1501–1520) and 5'-CCA GCT TTG GTG GGG TCA A-3' (antisense, bp 1953–1934); the final PCR product was 453 bp in size. The PCR products were separated on a 1.5% agarose gel in 1x TBE buffer, containing 0.09 M Tris borate and 0.002 M EDTA, pH 8.0 (10 V/cm gel length for 1 h), stained with ethidium bromide (0.5 µg/ml), visualized under ultraviolet light, and photographed.

The RT-PCR products for CAT1, CAT2A, CAT2B, and CAT3 were ligated into pCR II vector (Invitrogen, San Diego, CA), and the subsequent plasmid DNA was purified using an exchange column (Qiagen, Chatsworth, CA). To confirm the authenticity of the RT-PCR product, each insert was sequenced with ThermoSequenase using the dideoxynucleotide chain termination reaction (Amersham). The samples were resolved on a DNA sequencer model 725 (Molecular Dynamics).

Statistical methods. Data are presented as means ± SE. The significance of differences in L-arginine transport or NOS activity was evaluated using a one-way ANOVA and a Tukey post hoc test. A confidence level of P < 0.05 was considered significant.

RESULTS

Characteristics of L-arginine transport. The uptake of 50 µmol/l L-[3H]arginine was linear up to 2 min of incubation time (r² = 0.99) but tended to decrease thereafter (n = 6, data not shown). The absolute rate of L-arginine uptake averaged 39.4 ± 6.5 pmol·mg⁻¹·min⁻¹ after 2 min. Substitution of sodium chloride in the uptake buffer with equimolar amounts of choline chloride had no effect on L-arginine uptake, indicating that L-arginine transport by IMCD cells is independent of sodium (n = 6). Subsequent experiments were therefore performed with uptake measured over a 1 min time period in an uptake buffer that contained sodium.

In the kinetic studies, saturable uptake of L-[3H]arginine (2–1,000 µmol/l) was measured. The plot of L-arginine uptake as a function of extracellular L-arginine concentration is shown in Fig. 1. Nonlinear regression of the Eadie-Hofstee transformation (Fig. 1, inset) demonstrated that the best fit was an exponential equation (V = 887e⁻⁰.⁷³x/5, r² = 0.99). These data indicated that L-arginine uptake in the isolated IMCD cell suspension was biphasic with high-affinity state [maximal velocity (Vₘₐₓ) = 193 pmol·mg⁻¹·min⁻¹, Vₐₙₜ]
The addition of excess L-arginine (n = 5) also led to a significant decrease in the uptake of radiolabeled L-arginine (P < 0.05, from control). d-Arginine (n = 7) also had a significant inhibitory effect on L-arginine transport (P < 0.05, from control). In contrast to the effects of cationic amino acids, the neutral amino acids L-leucine (n = 7), L-glutamine (n = 5), L-serine (n = 6), α-(methylamino)isobutyric acid (MeAIB, a test substrate for system A, n = 5), and 2-amino-2-norbornanecarboxylic acid (BCH, a test substrate for system L, n = 5) did not inhibit L-arginine uptake by isolated IMCD. The L-arginine analogs known to be potent inhibitors of NOS exhibited diverse effects on L-arginine transport. N⁵-monomethyl-L-arginine (L-NMMA, n = 5) blocked L-arginine uptake (P < 0.05, from control), but neither L-NAME (n = 7) nor N⁵-nitro-L-arginine (L-NNA, n = 8) altered L-arginine uptake by IMCD. The inhibition profile is consistent with system y* as the predominant L-arginine transport system in IMCD cells.

L-Arginine transport and NOS activity. In these experiments the reaction period was extended to 5 min to measure both L-arginine transport and NOS enzymatic activity in IMCD cells. This extended reaction time was necessary to accurately quantitate the formation of L-citrulline from L-arginine. The control values for L-arginine uptake and NOS activity by the intact cells averaged 34.4 ± 1.5 pmol·mg⁻¹·min⁻¹ and 23.8 ± 3.9 pmol·L-citrulline·mg⁻¹·min⁻¹, respectively. The changes in L-arginine transport and NOS enzymatic activity, expressed as a percentage of control values, following the addition of different amino acids are illustrated in Fig. 3. The addition of the cationic amino acids L-lysine and L-ornithine or the arginine analog L-NMMA all led to a dose-dependent decrease in both L-arginine uptake and in NOS activity. Though L-NMMA directly inhibits NOS enzymatic activity independent of effects on L-arginine transport, neither L-lysine (19) nor L-ornithine (unpublished data) directly alter NOS enzymatic activity in cell homogenates. These results therefore indicate that L-arginine uptake is necessary for NO production under the present experimental conditions.

Identification of CAT isoforms in microdissected IMCD. To identify the y⁺ transporter present in the IMCD, RT-PCR amplification was performed on RNA obtained from microdissected IMCD. Parallel reactions were performed on RNA obtained from rat liver, brain, and kidney to serve as positive (and negative) controls for the different RT-PCR primer pairs. The sizes of the CAT1, CAT2A, CAT2B, and CAT3 RT-PCR products were 650, 1,160, 1,161, and 453 bp, respectively. As shown in Fig. 4, RT-PCR products for CAT1 were detected in the brain and kidney; CAT2A was detected only in liver RNA; CAT2B was detected in the liver, brain, and kidney; and CAT3 was found in both the brain and kidney. Interestingly, in the microdissected IMCD, the only transcript detected was CAT1 (Fig. 4, bottom). No PCR products were obtained from IMCD RNA samples when the RT reaction was eliminated, demonstrating that the RT-PCR products were not derived from genomic DNA.

DISCUSSION

Recent work from our laboratory has focused on the action of NOS in the regulation of renal/cardiovascular function. It has been observed that the renal medulla, in particular the IMCD, possesses a large amount of NOS enzymatic activity (41). In addition, inhibition of NOS in the renal medulla leads to the retention of sodium and water and the development of hypertension (23). To begin to examine the regulation of NO production in the renal medulla, the present studies were designed to characterize the pathway of cellular uptake of L-arginine and to determine the influence of cellular L-arginine uptake on NOS enzymatic activity in a suspension of intact IMCD cells. These experiments demonstrate that L-arginine uptake in IMCD is predominantly mediated by a high-affinity y⁺ transporter system encoded by CAT1. Blockade of this transporter with other cationic amino acids resulted in a significant decrease in L-citrulline formation, indicating that NO production by NOS in these cells is dependent on cellular L-arginine uptake. The results of this study therefore indicate that extracellular L-arginine levels and the cellular transport mechanisms may be impor-
The kinetics of L-arginine uptake in IMCD and the inhibition profiles of L-arginine by other amino acids indicate that the predominant transporter of L-arginine transport into IMCD is system y\(^+\). This assignment is based on the observations that this transport is saturable, sodium-independent, not inhibited by neutral amino acids (L-leucine, L-glutamine, and L-serine), and blocked by other cationic amino acids (L-lysine, L-ornithine, and L-homoarginine) (22). Furthermore, the addition of MeAIB (a test substrate for system A) or BCH (a test substrate for system L) did not significantly alter L-arginine uptake. The kinetic studies of Na\(^+\)-independent L-arginine transport revealed the presence of a higher affinity (K_{m} = 128 µmol/l) L-arginine transporter. This is consistent with previous work that characterized a high-affinity system y\(^+\) carrier in cultured endothelial cells (106–140 µmol/l) (2, 36), cardiac myocytes (125 µmol/l) (35), vascular smooth cells (110 µmol/l) (10), and neurons (47–88 µmol/l) (37, 40).

L-Arginine analogs possessing an altered guanidino nitrogen atom, such as L-NAME, L-NNA, and L-NMMA, are potent inhibitors of NOS. However, they have divergent effects on L-arginine uptake. L-NMMA bearing a basic guanidino group effectively inhibited the transport of L-arginine by IMCD cells, whereas the neutral analogs L-NNA and L-NAME had no influence on L-arginine transport. This finding is consistent with previous reports obtained with cultured endothelial cells, vascular smooth cells, and neurons (10, 32, 36, 40) and suggest that the cellular uptake of these L-arginine derivatives is mediated by different transport systems. Furthermore, the ability of L-NMMA to block L-arginine transport into cells may provide an additional mechanism by which L-NMMA can block NO production. The use of compounds that compete with L-arginine for the transporter may be an alternative strategy for control of NO production in intact cells under experimental circumstances.

Following the observation that large amounts of NOS enzymatic activity are present in certain segments of the nephron, the control of NO production in these cells is a question of great experimental interest. One possible controller of NO production by NOS in IMCD is the intracellular availability of L-arginine. Most mammalian cells obtain L-arginine from extracellular sources (7, 22). Thus levels of intracellular L-arginine and NO production will be highly sensitive to the supply of exogenous L-arginine, a process that can be modified by L-arginine transport inhibitors. The present observation that compounds which block L-arginine uptake effectively inhibit NO production by IMCD suggests that L-arginine transport may be a rate-limiting step in NO synthesis by IMCD cells and that L-arginine uptake inhibitors by themselves can be effective inhibitors of NO production.

It is not presently clear whether cellular L-arginine uptake is a limiting factor in the regulation of renal function under normal circumstances. The extracellular concentration of L-arginine normally ranges from 100–200 µmol/l, which would be predicted to provide ample substrate for the NOS isoforms, which possess reported K_{m} values less than 10 µmol/l (12). One site in the body in which extracellular L-arginine may be limited could be the renal medulla. Although cells of the renal cortex, particularly the initial segments of the proximal tubules, contain the enzymatic machinery capable of synthesizing L-arginine (18), the fractional excretion of L-arginine in rats is ~0.03% of filtered load, indicating that tubular concentration of L-arginine in the medullary collecting duct may be extremely low (33). Furthermore, although the renal medullary interstitial concentration of L-arginine is not known, total tissue L-arginine (normalized to wet weight or total cellular protein) is three to four times lower in the papilla than in the renal cortex (34, 41). Finally, it has been demonstrated that NO levels in the renal medullary interstitial space double following the intravenous infusion of L-arginine, indicating that NOS in the renal medulla may indeed be substrate limited (43). This subject will require further study to begin to fully understand the role of extracellular L-arginine in the regulation of NO formation in the renal medulla.

Uptake of extracellular L-arginine may be unnecessary for NO production by IMCD cells if L-arginine can be generated within IMCD. Some renal cell types have been demonstrated to be capable of generating L-arginine from L-citrulline by the sequential action of

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**Fig. 4.** RT-PCR products of cationic amino acid transporter isoforms CAT1, CAT2A, CAT2B, and CAT3 in microdissected IMCD cells. RT-PCR analysis of total RNA from rat liver, brain, and whole kidney was carried out in parallel to verify the authenticity and efficiency of CAT primer pairs. The sizes of the RT-PCR products of CAT1, CAT2A, CAT2B, and CAT3 were 650, 1,160, 1,161, and 453 bp, respectively. These results are typical of five independent observations.
argininosuccinate synthase and lyase (8, 18). Biochemical studies at the level of whole homogenized rat renal tissue, however, have demonstrated that argininosuccinate synthase and lyase exist in relatively low levels in the renal inner medulla (8). Further experiments in isolated IMCD have demonstrated L-arginine synthetic activity is not detectable in the IMCD (18). Under the in vitro conditions of the present study, administration of activity is not detectable in the IMCD (18). Under the in vitro conditions of the present study, administration of L-arginine for the CAT1 transporter lead to a concentration-dependent decrease in NO production in the isolated IMCD. This finding indicates that NO production by IMCD is dependent upon cellular L-arginine uptake, a concept which sharply contrasts with the observations previously reported in endothelial cells. It has been demonstrated that the enzymatic mechanisms necessary to recycle L-citrulline to L-arginine are present in endothelial cells (13, 42) and that endothelial cells are capable of sustaining the intracellular L-arginine levels despite continuous release of NO (24). The present data, along with previous biochemical studies, indicate that intracellular L-arginine concentration is indeed regulated by the rate of cellular L-arginine uptake in the IMCD. This may be an important mechanism whereby NO release is differentially regulated in epithelial and endothelial cells.

In summary, results of the present study demonstrate that the IMCD of the Sprague-Dawley rat kidney bears a Na+-dependent, saturable, L-arginine transport system with transport properties consistent with the y+ system. RT-PCR of microdissected IMCD cells revealed that the expression in IMCD is the CAT1 transporter. Furthermore, the data indicate that NO production by the IMCD is dependent on the cellular uptake of L-arginine. Since relatively large amounts of NOS are expressed in the IMCD and pharmacological blockade of NOS in the renal medulla has a profound influence on sodium and water excretion, this transport mechanism may play a key role in the regulation of fluid and electrolyte homeostasis and arterial blood pressure.

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