Original Paper

Arginine Transport Is Augmented, through Modulation of Cationic Amino Acid Transporter-1, in Obstructive Uropathy in Rats

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Abstract
Background: The decrease in glomerular filtration rate (GFR), which is characteristic of obstructive uropathy, was suggested to be associated with attenuated nitric oxide (NO) generation. Since availability of L-arginine, the sole precursor for NO, governs NO synthesis, we aimed to determine the role of glomerular arginine transport in rats subjected to 24 h of bilateral ureteral ligation (BUO). **Methods:** Glomerular arginine transport was measured by uptake of radiolabeled arginine ($[^3H]$-L-arginine), cationic amino acid transporters (CAT)-1 and -2 and arginases I and II mRNA expression were determined using reverse transcription-polymerase chain reaction. CAT-1, arginase I, and arginase II protein contents were evaluated by Western blotting. **Results:** L-Arginine transport by freshly harvested glomeruli from BUO rats was significantly augmented than in controls. The aforementioned findings were associated with a significant increase in glomerular CAT-1 mRNA expression, while CAT-2 mRNA was unchanged. Western blotting demonstrated a significant increase in CAT-1 abundance in BUO. Expression of both glomerular arginase I and II mRNA and protein content were significantly elevated in BUO. **Conclusions:** BUO induces an increase in glomerular arginine transport via upregulation of CAT-1, probably due to increase in arginine utilization by a non-NO pathway.

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Introduction

Obstructive nephropathy refers to the renal disease resulting from impaired flow of urine or tubular fluid as a consequence of structural or functional abnormalities of the urinary tract. Most experimental studies on obstructive uropathy have examined the effect of a short-term (<36 h) bilateral ureteral obstruction (BUO). A unilateral release of BUO of 24 h duration results in an increased renal vascular resistance. This may account, at least in part, for the marked decrease in glomerular filtration rate (GFR) and effective renal plasma flow observed in this setting [1]. The role of several vasoactive substances in renal hemodynamic alteration in this model has

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been studied [2, 3]. Klahr's group [4] have shown that administration of L-arginine prior to obstruction markedly increased GFR after release of obstruction, suggesting a role for L-arginine-nitric oxide (NO) system in the altered GFR observed in this model. They hypothesized that decreased delivery of L-arginine to endothelial cells in BUO may be the mechanism to provoke a decrease in GFR observed in their studies.

Among several transport systems which mediate L-arginine uptake (y+, $b^{0,+}$, $B^{0,+}$, and $y^+ L$), system y+ is widely expressed and considered to be a major arginine transporter in most tissues and cells. Encoded by cationic amino acid transporters (CAT)-1, CAT-2 and CAT-3, system y+ is characterized by high affinity for cationic amino acids, sodium independence, and stimulation of transport by substrate on the opposite (trans) side of the membrane [5, 6]. Accumulated evidence from our laboratory and others suggest that each transporter has affinity to a specific NOS isoform. We have previously shown that arginine uptake by either tubules or glomeruli harvested from rats subjected to ischemia and reperfusion (I/R), or to sepsis, two experimental models characterized by activation of the inducible NOS (iNOS), exhibit augmented arginine uptake, associated with upregulation of CAT-2 [7, 8]. In contrast, we have found that increased arginine uptake through upregulation of CAT-1 may contribute to the pathogenesis of diabetic hyperfiltration, via activation of eNOS [9]. Schafer et al. [10] have shown that suppression of the endothelium-mediated microvascular vasodilation by dexamethasone involves downregulation of eNOS and CAT-1. Moreover, CAT-1 and eNOS were found to be co-localized in a caveolar complex [11], therefore, this complex has been suggested to serve as a mechanism for channeling of newly acquired extracellular arginine to eNOS, for NO synthesis. Taken together, it is believed that arginine is delivered to eNOS predominantly by CAT-1.

The experiments described herein were designed to determine whether glomerular arginine uptake is altered in BUO, thus inducing an NO-deficient state, and to elucidate a molecular mechanism to explain these observations.

Methods

Materials

All standard reagents were obtained from Sigma (St. Louis Mo., USA), unless stated otherwise. [^{3}H]-L-arginine was supplied by PerkinElmer (Life and Analytical Sciences, Boston, Mass., USA).

Animals and Surgical Preparation

All animal experiments described in this study were conducted according to the Guide of Care and Use of Animals Protocol approved by the Institutional Committee on Ethics in Animal Experiments. Studies were performed using male Wistar rats at 12–14 weeks of age. BUO was performed by ligation of both ureters at the junction of their lower third and upper two thirds through a small suprapubic incision. The rats were returned to their cages and were not allowed food or water. All experiments were performed 24 h following surgery. Sham operations were performed in control animals.

Isolation of Glomeruli

Kidneys from all experimental groups were decapsulated, bisected and the cortex was carefully dissected free. Glomeruli were prepared using a sieving technique. Cortices were minced to a fine paste with a razorblade and gently pressed through a 106-μM stainless steel sieve. The resulting material was suspended in HEPES buffer (KCl 5 mM, CaCl$_2$ 0.9 mM, MgCl$_2$ 1 mM, D-glucose 5.6 mM, Hepes 25 mM, NaCl 140 mM), at 40°C, pH 7.4. The suspension was forced through a 20-gauge needle to decapsulate the glomeruli and then passed through a 75-μm sieve. The glomeruli which were trapped on the sieve were washed and pelleted by centrifugation at 1,000 rpm for 1 min. This was repeated 3 times. The fraction consisted of more than 95% glomeruli, the majority of which were decapsulated. Isolated glomeruli were used for arginine uptake assessments, RNA and protein extraction.

### L-Arginine Uptake in Freshly Harvested Glomeruli

L-Arginine uptake was determined essentially as previously described [8]. Glomerular suspensions from the various experimental groups were incubated and shaken for 10 min in HEPES-buffer, pH 7.4, 37°C. L-[^{3}H]-arginine and L-arginine, in a final concentration of 1 mM, were added to a total volume of 1 ml for additional 4 min. The duration of 4 min was chosen since it was within the linear portion of uptake curves (data not shown). Transport activity was terminated by rapidly washing the glomeruli with ice-cold PBS buffer (4 times, 2 ml/tube). The glomeruli were then dried and solubilized in 1 ml of 0.5% SDS in 0.5 N NaOH. 700 μl of the extract were used to monitor radioactivity, by liquid scintillation spectrometry (Betamatic, Kontron). The remaining 300 μl were used for protein content determination by using Lowry method. To correct for non-specific uptake or cell membrane binding, glomeruli were incubated with 10 mM unlabeled arginine in HEPES buffer, and the associated radioactivity was subtracted from each data point. The results are expressed as mean ± SE of at least 5 different experiments.

Analysis of mRNA Levels by Reverse Transcription-Polymerase Chain Reaction

Total cellular RNA was extracted from glomeruli following the method described by Chomeczynski and Sacchi [12]. Reverse transcription was carried out for 1.5 h at 42°C, and PCR in 1× Jeffrey's buffer [13], for 35 cycles, each of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and 7 min (final cycle). The first pair of primers was designed to bind to a portion of the rat CAT-1 gene: forward 21-mer, 5′-GCCATCGTCATCTCCTTCCTG-3′ and reverse 21-mer, 5′-CCCTCCCTCACCGTATTTCAC-3′ expression. A second pair of primers, which hybridize to a sequence, common to both CAT-2 and CAT-2A were: forward 24-mer, 5′-AACGT-

Fig. 1. Uptake of radiolabeled arginine ([³H]-L-arginine) by freshly harvested glomeruli from the various experimental groups. Data are presented as the mean ± SEM of at least 5 different experiments. * p < 0.05 vs. control. CTL = Sham operated rats, BUO = bilateral ureteral obstruction.
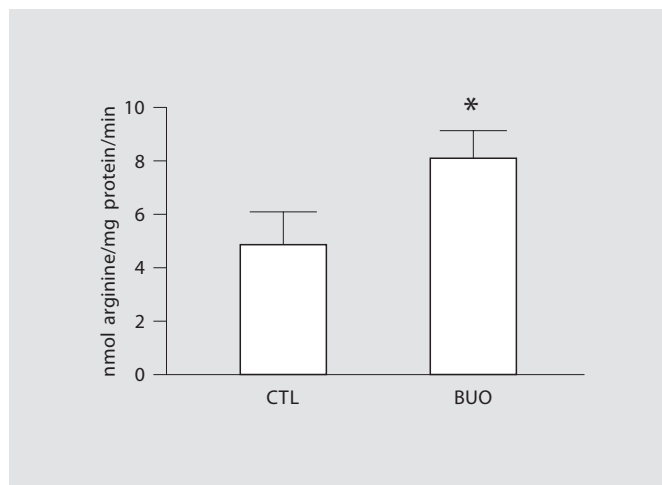
GCTTTTATGCCTTTGT-3′ and reverse 23-mer 5′-GGTGACCT-GGGACTCGCTCTT-3′ [14]. The following pairs of primers were used for arginase I: forward 25-mer, 5′-AAA GCC CAT AGA GAT TAT CGG AGC G-3′, and reverse 22-mer, 5′-AGA CAA GGT CAA CGG CAC TGC C-3′. For arginase II: forward 23-mer, 5′-TTA GTA GAG CTG TGT CAG GTG GC-3′ and reverse 24-mer, 5′-ACT TGA AGC AAT CAC ATC CAC TGC-3′ [9]. To exclude the possibility of contamination by genomic DNA amplification, and to asses the adequacy of cDNA, experiments were carried out in the absence of reverse transcriptase, and amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed, respectively. PCR products were electrophoresed on a 1.6% agarose gel and visualized by UV-induced fluorescence. All PCR reactions resulted in the amplification of a single product of the predicted size for CAT-1, CAT-2, arginase I, arginase II, and GAPDH.

Protein Quantification by Western Blotting

Glomerular CAT-1, arginase I and arginase II were determined by immunoblotting. Briefly, glomeruli were separately placed in ice-cold PBS lysis buffer (pH 7.4), containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 4.5 μM leupeptin, and 5 μM aprotinin) (ICN Biomedicals, Inc.), 0.01% Triton X-100 and 0.1% SDS, then mechanically homogenized and left on ice for 45 min. Homogenates were subsequently centrifuged (13,000 rpm for 10 min, at 4°C). Cell lysates were stored in aliquots in –70°C. A membrane fraction was obtained by adding to the pellet an equal volume of lysis buffer supplemented by Tween-20 (0.25%) to solubilize. The protein content of each sample was determined by the method of Lowry. Equal amounts of protein (30 μg) were prepared in sample buffer (2% SDS, 0.01% bromophenol blue, 25% glycerol, 0.0625 M Tris-HCl, pH 6.8, 5% mercaptoethanol) and analyzed on a 7.5% SDS-PAGE gel. The gel was transferred onto Hybond ECL nitrocellulose membranes (Amersham Corp.), and blocked in PBS-T containing 5% non-fat dried milk, at room temperature. Membranes were then incubated with polyclonal rabbit anti-rat CAT-1 antibodies (synthesized by Dr. O. Leitner, Weizmann Institute, Rehovot, Israel) and mouse anti-rat arginase I and II antibodies (both from Santa

Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) for 1 h at room temperature, washed, and incubated with secondary HRP-conjugated goat anti-rabbit antibody (1:10,000) in PBS-T for 1 h. Membranes were subsequently washed 3 times, for 5 min each, in PBS-T. Membranes were then stripped and reprobed with monoclonal anti-β-actin antibodies as an internal control. The reactive bands corresponding to CAT-1 were detected by enhanced chemiluminescence (Kodak X-Omat AR film) and quantified by densitometry.

Statistical Analysis

Data are presented as mean ± SE. One-way analysis of variance (ANOVA) was conducted for comparison between groups. Post-hoc analysis using an LSD algorithm was performed to allocate the source of significance.

Results

The first set of experiments was designed to explore a possible effect of BUO on the glomerular y+ system. Interestingly, when compared to sham operated rats, BUO induced a significant increase in glomerular arginine uptake (fig. 1). To determine whether the observed BUO-induced changes in arginine uptake are associated with similar directional changes in mRNA levels for CAT family of transporters, total glomerular RNA was analyzed by reverse transcription-coupled PCR to amplify portions of CAT-1 and CAT-2. When compared to sham operated animals, glomerular CAT-1 mRNA expression was significantly increased while CAT-2 remained unchanged (fig. 2). To determine whether the BUO-induced changes in CAT-1 mRNA expression are associated with parallel directional changes in CAT-1 protein content, Western blotting was performed. CAT-1 protein was
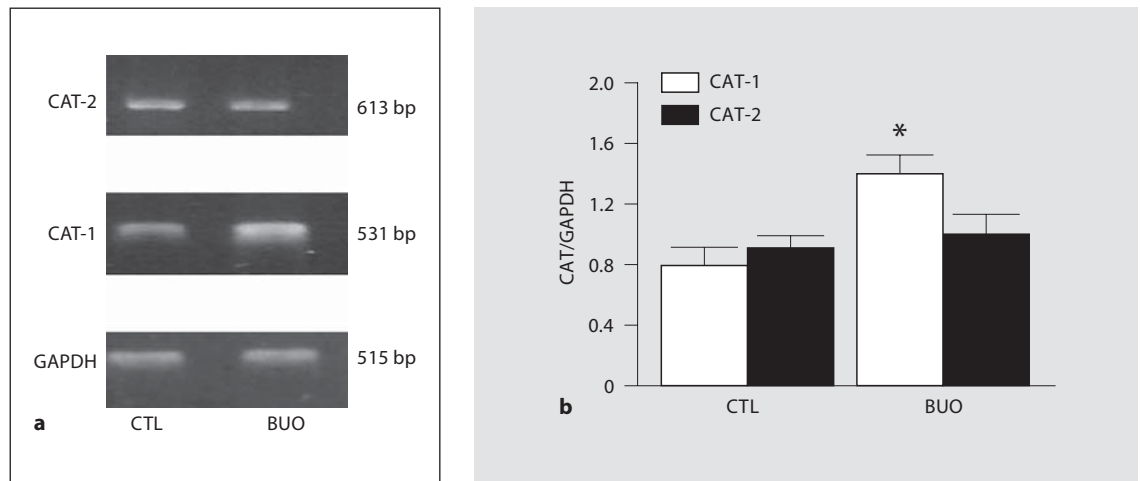
Fig. 2. a Ethidium-stained agarose electrophoresis gel showing PCR-amplified CAT-1, CAT-2, and GAPDH cDNA of glomeruli harvested from control (CTL) and rats with obstructive uropathy (BUO). These blots are representative of 3 different experiments. See text for definition of groups. **b** Relative amount of CAT-1 and CAT-2 mRNA quantitated by densitometry and expressed as CAT/GAPDH ratio of the same experiments shown in **a**. Data are presented as the mean ± SEM of 3 different experiments. * $p < 0.05$ vs. control.
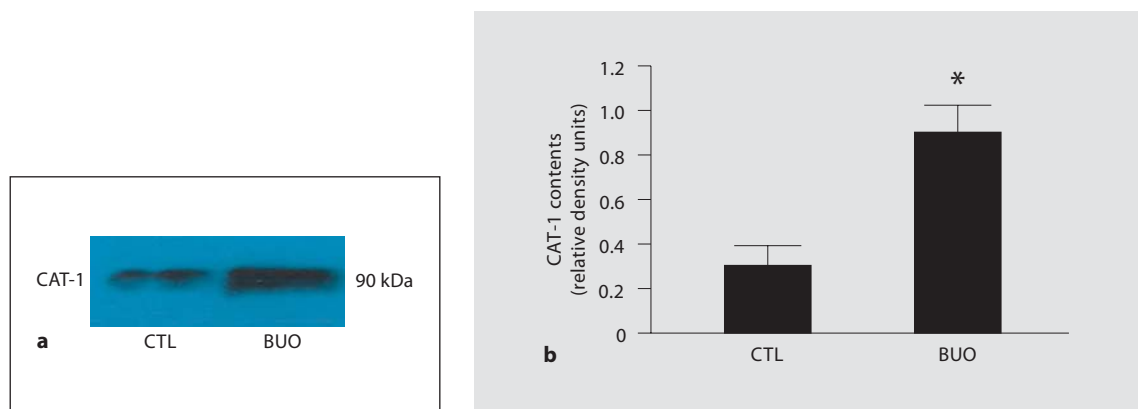

Fig. 3. a Representative Western blot analysis showing regulation of CAT-1 protein level in freshly harvested glomeruli from the same experimental groups. **b** Densitometric analysis of aortic CAT-1 contents from the various experimental groups. Each bar represents the mean of the relative density units ± SE from 3 different experiments, * $p < 0.05$ vs. control. CTL = Sham operated rats, BUO = bilateral ureteral ligation.

identified as ~90 kDa. We found that CAT-1 abundance was significantly increased in glomeruli harvested from BUO rats as compared to sham operated animals (fig. 3). To explore an alternative, non-NO pathway which can potentially utilize excess arginine, steady-state arginase I and arginase II were examined. We found that in BUO glomerular mRNA of both isoenzymes were significant-

ly augmented as compared to sham operated rats (fig. 4). To further explore a possible role for arginases in arginine traffic in BUO, we examined arginase I and II protein levels. We found that both arginase I and II protein abundance were significantly increased in glomeruli harvested from BUO rats (fig. 5).
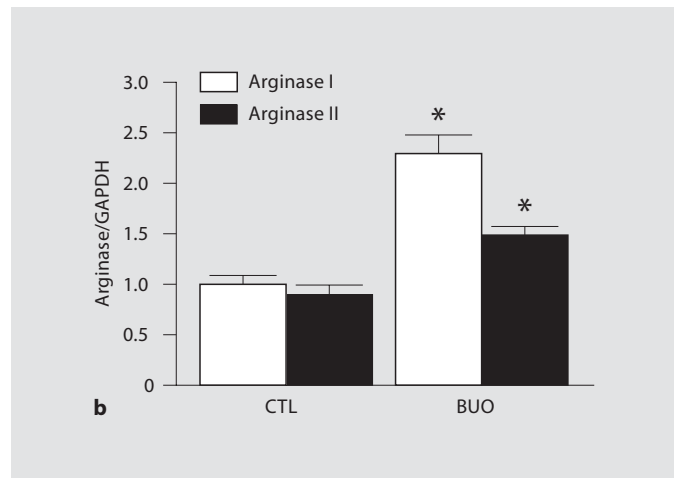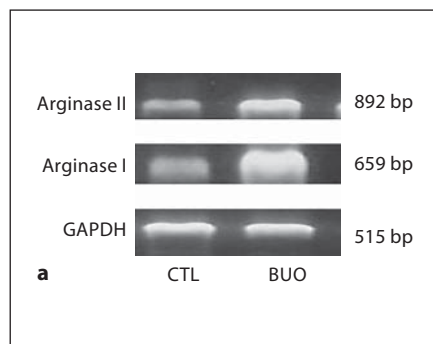
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Fig. 4. a Ethidium-stained agarose electrophoresis gel showing PCR-amplified arginase I, arginase II, and GAPDH cDNA from freshly harvested glomeruli of control and rats with obstructive uropathy. **b** Relative amount of arginase I and arginase II mRNA quantitated by densitometry and expressed as arginase/GAPDH ratio of the same experiments shown in figure 3a. Data are presented as the mean ± SEM of 3 different experiments. * $p < 0.05$ vs. control.
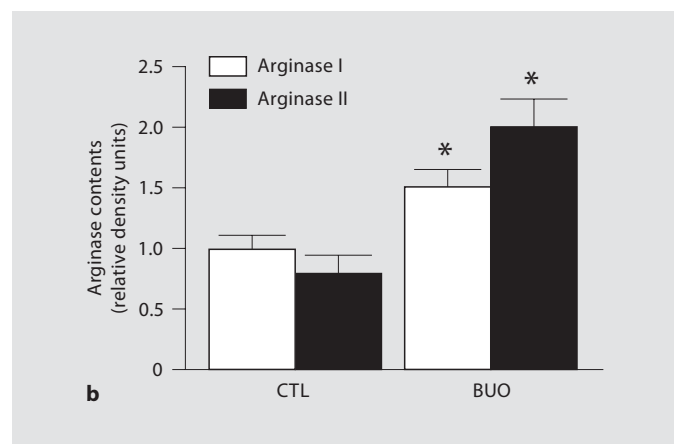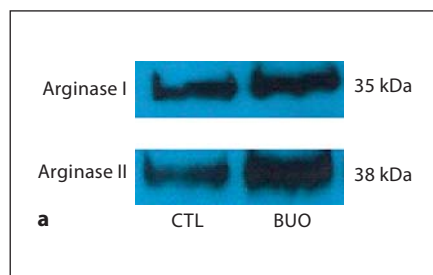

Fig. 5. a Representative Western blot analysis showing regulation of aortic arginase I and arginase II protein levels in freshly harvested glomeruli from the same experimental groups. **b** Densitometric analysis of aortic arginase I and arginase II contents from the various experimental groups. Each bar represents the mean of the relative density units ± SE from 3 different experiments, * $p < 0.05$ vs. control. CTL = Sham operated rats, BUO = bilateral ureteral ligation.

Discussion

The present study demonstrates that glomerular arginine uptake is increased in an in vivo model of obstructive uropathy. These findings contradict the hypothesis that in obstructive uropathy, arginine depletion downregulates NOS, thereby inducing renal vasoconstriction

[4]. We have previously shown in different experimental models of renal failure that arginine transport matches NOS activity. In other words, a decrease in NOS activity is accompanied by attenuation of arginine uptake by its specific arginine transporter and vice versa. For example, in uremic rats, eNOS inactivation is associated with attenuated arginine uptake due to a decrease in CAT-1 pro-

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tein content [15]. On the other hand, glomeruli harvested from rats subjected to I/R or to sepsis, two experimental models characterized by activation of iNOS exhibit augmented arginine uptake, associated with upregulation of CAT-2 [7, 8]. Nicholson et al. [16] have reported that iNOS activity was reduced in macrophages from CAT-2 knockout mice, implying that upon induction of iNOS the excess arginine required is delivered by CAT-2. In the current studies arginine transport was found to be increased through modulation of CAT-1, the selective eNOS arginine supplier, while CAT-2 mRNA expression was unchanged. Our findings are not in accord with the hypothesis suggested by Klahr et al. [1], and cannot be linked to glomerular iNOS induction as reported by Moridaira et al. [17] in a similar experimental model. Therefore, it is conceivable to hypothesize that, in obstructive uropathy, arginine is diverted towards other pathways which are not directly associated with NO generation. Only a small fraction of cellular L-arginine is utilized for NO synthesis. In addition to NO generation, L-arginine is utilized during synthesis of creatine by L-arginine glycine amidinotransferase, agmatine by arginine decarboxylase, and ornithine by arginase [18]. We wished therefore to examine whether the increase in glomerular arginine transport is associated with activation of a non-NO arginine pathway. Indeed, we found that mRNA expression and protein content of both arginase I and II are upregulated. These data suggest that in BUO, the increase in arginine transport is diverted towards increased generation of polyamines or utilization by the urea cycle. One can spec-

ulate that activation of the aforementioned pathways may attenuate NO generation. Arginases compete with NOS for L-arginine and can therefore limit NO production. Indeed, in endothelial cells overexpressing arginase I and II, NO synthesis has been shown to be attenuated, and inhibition of arginases restores endothelial NO synthesis [19, 20]. The fact that the changes observed in arginine uptake were associated exclusively with CAT-1 expression deserves future studies, to explore a possible association between non-NO arginine pathways to define arginine transporters. Specifically, does arginase activation involve extracellular arginine utilization through CAT family of proteins? This conclusion bears a limitation as the exclusivity of CAT-1 was not fully established since we were unable to follow CAT-2 protein.

Do upregulation of arginase isoforms play a role in the pathogenesis of obstructive uropathy? Matsuda et al. [21] have shown that glomeruli from rats subjected to BUO exhibited morphological changes which resemble those observed in experimentally induced hyperfiltration and polyamines have been suggested to play a role in diabetes-induced kidney growth [22]. One can speculate that, in BUO, diversion of arginine through arginase activation may promote glomerular hypertrophy via increased polyamines synthesis.

In conclusion, ureteral obstruction induces an increase in glomerular arginine transport via upregulation of CAT-1 probably for arginine utilization by a non-NO pathway.

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