Uremic levels of urea inhibit L-arginine transport in cultured endothelial cells

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Xiao, Shen, Laszlo Wagner, James Mahaney, and Chris Baylis. Uremic levels of urea inhibit L-arginine transport in cultured endothelial cells. Am J Physiol Renal Physiol 280: F989–F995, 2001.—Hypertension in end-stage renal disease (ESRD) may involve lack of endothelial nitric oxide (NO), as suggested by reduced total NO synthase (NOS) in dialysis patients. One reason might be due to substrate deficiency. To test the hypothesis that uremia is a state of intracellular L-arginine deficiency, uremic plasma was obtained from dialysis patients, and its effect was tested on L-arginine transport. Over a 6-h period, urea-induced inhibition of L-arginine transport was significant in human dermal microvascular endothelial cells (HDMEC) incubated for 6 h with 20% uremic plasma from peritoneal dialysis and hemodialysis patients obtained immediately predialysis. Similar transport inhibition was seen with ESRD plasma in human glomerular capillary and bovine aortic endothelial cells. Hemodialysis partially reversed inhibition of L-arginine transport. HDMECs incubated for 6 h with synthetic media containing high (uremic) urea concentrations showed inhibition of L-arginine transport, but this was not competitive because acute exposure to urea had no impact on L-arginine transport. Over a 6-h period, urea-induced inhibition of L-arginine transport was not sufficient to inhibit NOS activity, but after 7 days NOS activity was reduced. These cellular findings suggest that substrate delivery may be lowered, thus reducing endothelial NOS activity and contributing to hypertension in ESRD patients.

Hypertension is a frequent complication of end-stage renal disease (ESRD). Deficiency of endothelial nitric oxide (eNO) has been implicated in some forms of hypertension (2, 12, 29), and we have reported reductions in total NO synthase (NOS; from 24-h NO2 + NO3 = NOx production) in both peritoneal dialysis (PD) and hemodialysis (HD) patients (27, 28). One possible mechanism of NO deficiency in ESRD could be a deficiency of the NOS substrate L-arginine. Patients with ESRD lack functional renal mass, and the major source of endogenous L-arginine used by NOS is derived predominantly from the normal kidney cortex above the Michaelis-Menten constant ($K_m$) of the eNOS enzyme (5). Thus ESRD should not be associated with substrate-dependent falls in NO production, unless plasma arginine levels do not reflect intracellular arginine availability, perhaps because of reduced arginine transport into endothelial cells.

We therefore conducted the present study to assess the effects of plasma obtained from ESRD patients and controls on L-arginine transport into cultured vascular endothelial cells. After determining that ESRD plasma does inhibit L-arginine transport, we subsequently studied the effects of various synthetic solutions on L-arginine transport, in an effort to isolate the uremic plasma constituent(s) responsible for inhibition of L-arginine transport. We found that 6 h of incubation with uremic levels of urea inhibited L-arginine transport. We then performed both short (6 h)- and long-term (7 days) chronic studies to investigate the impact of a uremic level of urea on several aspects of NOS in cultured endothelial cells to produce NO. The majority of the studies were conducted on human dermal microvascular endothelial cells, but some experiments were on human glomerular endothelial cells and on bovine thoracic aortic endothelium.

**METHODS**

Characteristics of the subjects who provided plasma are given in Table 1, and further information is given elsewhere (27, 28). All hemodialysis patients were dialyzed with polysulfone membranes on high-flux F-80 dialyzers (Fresenius, Lexington, MA). All blood samples were harvested into heparin-coated tubes, spun cold, aliquotted, frozen within 20 min of collection, and then thawed immediately before use. Studies were conducted on human dermal microvascular endothelial cells (HDMEC; Clonetics, San Diego, CA), human glomerular endothelial cells (HGE; Cell System, Kirkland, WA), and bovine aortic endothelial cells (BAEC; established in our laboratory). Experiments were on HDMEC (passage 4–7), HGEs (passage 4–7), and BAECs (passage 2–4) (see Ref. 32 for details on growth media and culture conditions). In preparation for experiments, cells were subcultured into 12-
well plates and, when just confluent, the culture medium was replaced with minimal essential medium (MEM) containing 20% human plasma (uremic plasma or normal control) or synthetic solutions for 6 h, unless otherwise stated, and were then studied for arginine transport. In some conditions, NOS activity was measured.

Transport of l-arginine into endothelial cells was measured by the method of Gazzola et al. (11) with minor modifications. Briefly, after incubation with test medium, cells were washed with Krebs-HEPES buffer, then 0.5 ml Krebs-HEPES buffer containing 50 μM l-arginine with 1 μCi l-[3H]arginine was added to each well. Transport was terminated by removing the media and washing the cells (3X) with ice-cold 10 mM unlabeled l-arginine in PBS. Cells were lysed with 0.5% Triton X-100 in 0.5 M NaOH, and radioactive l-arginine was assayed by liquid scintillation counting. Background radioactivity was determined by transiently exposing cells to Krebs-HEPES buffer containing 10 mM unlabeled l-arginine + 1 μCi l-[3H]arginine and was subtracted from each data point. NOS activity was determined by measuring l-[3H]arginine conversion to l-[3H]citrulline over 60 min, according to the method of Davda et al. (8), with minor modifications, as described by us previously (33). The total cellular protein was determined by the Bio-Rad detergent method, which uses a modification of the Lowry assay (21) with BSA as a standard.

Pilot time course experiments showed that l-arginine uptake by endothelial cells increased linearly during the first 3 min in HDMECs and HGECs and the first 5 min in BAECs (data not shown). Therefore, measurement of l-arginine transport was performed 3 min after the addition of l-[3H]arginine. We routinely conducted parallel studies using Nω-nitro-l-arginine (l-NMA; 2 mM), a competitive inhibitor of l-arginine for the cationic amino acid transporter (CAT) family of membrane transporters (4).

The first series of experiments used 20% human plasma from normal subjects or ESRD patients for 6-h incubation with all three types of endothelial cells to determine the impact on arginine transport. Subsequent experiments were conducted on HDMECs grown in normal cell culture medium (33) with various synthetic solutions. We first used synthetic solutions to determine whether uremic constituents had an immediate, competitive inhibitory action on endothelial cell l-arginine transport. We used l-NMA and also Nω-nitro-l-arginine methyl ester (l-NNAME), 2 mM, which is transported by a different arginine transporter, not a member of the CAT family (4); the endogenous methylated arginine, asymmetric dimethylarginine (ADMA; 2.5, 10, and 100 μM), d-arginine (2 mM), and the uremic marker urea (25 mM). In addition, the CAT lysine (2 mM) was used as a competitive inhibitor of the CAT transporter. These various agents were added directly into the assay buffer (not preincubated) to test for competitive inhibition of arginine transport.

In addition, an extensive series of studies to measure l-arginine transport and sometimes NOS activity were done with synthetic solutions, which were preincubated with the cells for 6 h. These solutions all contained (in mM) 117 NaCl, 5.4 KCl, 1.8 CaCl2, 0.8 MgSO4, 26.2 NaHCO3, 1.0 NaH2PO4, 5.5 d-glucose, and 20% FCS. Some studies involved the addition of uremic levels of ADMA (2.5 μM); however, the majority involved different concentrations of urea. "Uremic" medium contained 50 m urea and 30.5 mM d-glucose (311 mosmol/l), and "control" medium contained an additional 50 mM d-glucose and no urea (308 mosmol/l). We also conducted a separate dose-response curve to urea with levels of 1, 5, 15, and 25 mM. Amino acids were added in some experiments to mimic the uremic plasma profile (in mM) 0.025 l-citrulline, 0.10 l-arginine, 1.0 l-glutamine, 0.2 l-lysine, 0.5 l-glycine, 0.1 mM l-cysteine) in the 50 mM urea solution or normal plasma amino acids (in mM) 0.05 l-citrulline, 0.10 l-arginine, 0.75 l-glutamine, 0.2 l-lysine, 0.25 l-glycine, 0.05 l-cysteine) in the 55.5 mM glucose solution (10).

In addition to studies on the effect of urea on l-arginine transport and eNOS activity after 6-h incubation, we also conducted chronic studies in HDMEC. Either 25 mM urea or 25 mM mannitol (as osmotic control) was added to the normal synthetic medium (containing 20% FCS) for 7 days (medium changed on alternate days), and l-arginine transport and eNOS activity were then measured.

In a final series, BAECs were grown in T75 flasks for measurement of total intracellular arginine concentration and eNOS protein abundance by Western blot. Confluent BAECs were incubated for 6 h with either 30.5 mM glucose or 25 mM urea + 5.5 mM glucose media. For chronic studies, BAECs were seeded into T75 flasks (~300,000 cells/flask), and 24 h later the medium was changed to contain either 25 mM urea or 25 mM mannitol (changed on alternate days) for the next 7 days. Arginine was measured on cell lysates by reverse-phase HPLC by using the AccQ Tag method, as described by us previously (27). The abundance of eNOS protein was measured on cell lysates by standard Western blot techniques by using the Transduction Laboratories mouse antibody N30020 at 1:1,000 dilution in TBS-T block room temperature (RT) and a secondary antibody, goat anti-mouse IgG-horseradish peroxidase (HRP), 1:2,000 dilution for 1 h at RT. Membranes were then stripped and reprobed with β-actin (Sigma mouse antibody A5441, 1:60,000 dilution for 1 h at RT; secondary antibody: goat anti-mouse IgG-HRP), 1:60,000 dilution for 1 h at RT. For additional details, see Mattson and Bellehumeur (15). Equal loading was also verified by Ponceau red staining of
the membrane, before probing with specific antibodies. The eNOS abundance was visualized by enhanced chemiluminescence (Kodak X-OMAT AR film was exposed to the membrane) and measured by image analysis (Optimas 6.2, Bothell, WA) of the integrated optical density, normalized for β-actin.

Arginine transport was expressed as picomoles arginine transported per minute per milligram of protein, and NOS activity, as picomoles arginine converted to citrulline per minute per milligram of protein. Individual numbers in each experiment were variable and are provided. Results are expressed as means ± SE. Statistical analysis was performed with the use of Student’s unpaired t-test or one-way ANOVA. Values of P < 0.05 are considered to be significantly different.

RESULTS

Figure 1 summarizes the effect of the various categories of human plasma on L-arginine transport into HDMECs (top). After 6-h incubation with 20% plasma from ESRD patients, the L-[3H]arginine uptake was significantly inhibited by both the PD and pre-HD plasma compared with control (P < 0.01), and there was no difference between the inhibitory effect of PD and pre-HD. Hemodialysis did remove some of the arginine transport inhibitory effect of uremic plasma, because the inhibition of L-arginine uptake was attenuated by post-HD plasma relative to pre-HD (P < 0.05) but remained below control (P < 0.05). On the basis of the characteristics of the dialysis membrane used, we would expect rapid clearance of low- and some middle-molecular-weight solutes. We observed similar patterns of inhibition of L-arginine transport in separate experiments in both HGECs and BAECs (Fig. 1). In all cases, the majority of L-arginine entry into the cell was inhibited by L-NMA in cells incubated in control and uremic plasma (open bars).

Figure 2 shows the acute effect of several agents on L-arginine transport. In HDMECs compared with L-NMA, L-NAME was completely ineffective in reducing L-arginine transport, which may reflect the fact that L-NAME uses a neutral amino acid transporter rather than one of the CAT family (4) and that this neutral transporter may be absent from cultured endothelial cells. ADMA did exert some inhibitory effect on L-arginine transport at a pharmacological concentration of 100 μM but not at lower concentrations of 2.5 or 10 μM, in the range seen in uremia. D-Arginine was without any inhibitory effect, but the CAT L-lysine competed with L-arginine for the membrane transporter, consistent with mem-

![Fig. 1. Effects of 20% human plasma from normal controls, patients on peritoneal dialysis (PD), both pre- and posthemodialysis (HD), on L-arginine transport in human dermal microvascular endothelial cells (HDMECs) after 6-h incubation. Measurements were made in the baseline state (solid histograms) and with 5 mM N^G-monomethyl-L-arginine (L-NMA) (open histograms). HGEC, human glomerular endothelial cells; BAEC, bovine aortic endothelial cells. Results are means ± SE of 3 separate experiments, each performed in triplicate. *P < 0.01 compared with control. #P < 0.05 compared with pre-HD and control.](http://ajprenal.physiology.org/)

![Fig. 2. Acute effects of the arginine analogs L-NMA (2 mM), N^G-nitro-L-arginine methyl ester (L-NAME; 2 mM), asymmetric dimethylarginine (ADMA; 2.5, 10, 100 μM), D-arg (2 mM), the amino acid L-lysine (2 mM), and urea (25 mM) on L-arginine transport in HDMEC. Results are means ± SE of 3 separate experiments, each performed in triplicate. *P < 0.05 compared with control.](http://ajprenal.physiology.org/)
brane transport via the CAT system (9). Urea (25 mM) had no acute effect on L-arginine transport.

Because the human plasma required 6-h preincubation to exert an effect on L-arginine transport, we conducted similar preincubation studies with synthetic solutions. Six-hour exposure to a uremic level of ADMA (2.5 mM) had no impact on L-arginine transport vs. control (294 ± 11 vs. 291 ± 20 pmol arginine transported·min⁻¹·mg protein⁻¹ and 17.4 ± 1.1 vs. 16.1 ± 1.1). There was no evidence that the total intracellular arginine level was altered by 6-h incubation with 25 mM urea, although after 7 days intracellular arginine was higher in urea treated vs. control cells, perhaps reflecting the decreased utilization by NOS. By Western blot analysis, the abundance of the eNOS enzyme (relative to β-actin) was unaffected by either 6-h or 7-day incubation with 25 mM urea vs. control (0.89 ± 0.11 vs. 0.89 ± 0.32 and 1.05 ± 0.35 vs. 1.07 ± 0.32 integrated optical density units, respectively). A representative blot is shown in Fig. 5.

DISCUSSION

Our studies demonstrate that uremic plasma contains arginine transport inhibitory material, because we observed substantial inhibition (∼20–30%) of L-arginine transport into three different types of cultured vascular endothelial cells, when incubated with ESRD patient plasma. The inhibitory factor(s) is present in plasma of patients on both PD and HD and thus probably results from the renal insufficiency, rather than the treatment modality. This observation may reconcile the apparent contradictory finding of only mildly subnormal values of plasma arginine concentration in ESRD, with the expectation of arginine deficiency, due to loss of renal arginine synthesis (19, 26, 27, 28). A reduced rate of L-arginine removal from plasma (and subsequently reduced utilization) would raise plasma arginine levels and camouflage a reduction in L-arginine availability. When we consider that BAECs after 6-h incubation with 25 mM urea, whereas eNOS activity in HDMECs was inhibited by 25 mM urea after 7 days. These effects on L-arginine transport and eNOS activity after 7-day incubation were due to urea (Fig. 3, Table 2) because similar changes in the cell medium osmolarity with mannitol did not alter L-arginine transport and NOS activity compared with control synthetic medium (228 ± 27 vs. 217 ± 18 pmol arginine transported·min⁻¹·mg protein⁻¹ and 17.4 ± 1.1 vs. 16.1 ± 1.1). There was no evidence that the total intracellular arginine level was altered by 6-h incubation with 25 mM urea, although after 7 days intracellular arginine was higher in urea treated vs. control cells, perhaps reflecting the decreased utilization by NOS. By Western blot analysis, the abundance of the eNOS enzyme (relative to β-actin) was unaffected by either 6-h or 7-day incubation with 25 mM urea vs. control (0.89 ± 0.11 vs. 0.89 ± 0.32 and 1.05 ± 0.35 vs. 1.07 ± 0.32 integrated optical density units, respectively). A representative blot is shown in Fig. 5.
both renal production of arginine and arginine transport into endothelial cells are reduced in ESRD, this suggests that the normal to mildly subnormal values of plasma arginine encountered in ESRD do indeed reflect an arginine deficiency. This view is supported by the observation that orotic acid levels (a marker for arginine deficiency) are increased in uremia (26).

The inhibitory agent is likely to be a small or middle-sized molecule because it is partially cleared during hemodialysis with polysulfone membranes. Previous studies in our laboratory and others have found that the endogenous L-arginine analog ADMA accumulates in renal failure in concentrations sufficient to inhibit NOS and is partially cleared by hemodialysis (1, 7, 14, 22, 27, 28, 30). Because ADMA is transported into endothelial cells by the CAT transport system, which is also the major route of endothelial L-arginine transport (9), we investigated whether a uremic level of ADMA might also cause transport inhibition. However, whereas uremic levels of ADMA (2–10 μM) can inhibit eNOS activity (33), we found that these levels are too low to affect L-arginine transport, because competitive inhibition was only seen at pharmacological concentrations of ADMA (100 μM).

Thus some other factor(s) in ESRD plasma must be responsible for the inhibition of L-arginine transport, and our studies with synthetic solutions indicate a role for urea. Urea is a waste end product of protein metabolism made from the transformation of L-arginine to ornithine by the action of arginase (19). Urea accumulates in renal failure, reaching ~5–10× normal concentrations at ESRD. Although conventionally regarded as innocuous, there is evidence that in high concentrations urea can inhibit the Na\(^+\)-K\(^+\) pump and a number of other enzymes and generally interfere with cellular metabolism (6, 13, 25). Our data with synthetic solutions show that a uremic level of 25 mM urea (~75 mg/dl blood urea nitrogen (BUN)) significantly inhibited L-arginine transport into HDMEC after 6-h incubation. Because we made similar observations in HGEC and BAEC, we consider that high extracellular urea has a general, widespread effect on L-arginine transport in vascular endothelial cells. Of note, there must be other transport inhibitory factors in uremia because we see substantial inhibition in vitro with a one-fifth dilution of human ESRD plasma, which is ~5 mM urea, whereas a synthetic solution of 5 mM urea has no impact on transport of L-arginine. We have some preliminary unpublished data suggesting that creatinine (10 mg/100 ml) also inhibits L-arginine transport after 6-h incubation, and other L-arginine transport inhibitory factors may also be present. Given the impact of the individual constituents of uremic plasma in vitro, it is possible that in vivo uremic plasma has more marked inhibitory effects on L-arginine uptake.

Earlier work suggested that urea competitively inhibits the action of several enzymes by disrupting the binding of substrate to enzyme (25). Our studies suggest that this is not a competitive inhibition for transporter sites because the addition of different concentrations of urea directly to the assay buffer had no acute effect on L-arginine transport. If urea gains entry

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**Table 2. Impact of uremic level of urea on NOS activity**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>6-h Preincubation</th>
<th>7-Day Preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (25 mM glucose)</td>
<td>25 mM urea</td>
</tr>
<tr>
<td>HDMEC</td>
<td>13.4 ± 1.9 (1.6 ± 0.2)</td>
<td>13.6 ± 1.8 (1.9 ± 0.3)</td>
</tr>
<tr>
<td>HGEC</td>
<td>16.4 ± 1.1 (2.4 ± 0.7)</td>
<td>14.9 ± 1.7 (2.6 ± 0.7)</td>
</tr>
<tr>
<td>BAEC</td>
<td>30.3 ± 2.1 (2.9 ± 1.0)</td>
<td>21.5 ± 2.4 (2.8 ± 0.8)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Values in parentheses are +2 mM. NOS activity is expressed as pmol arginine converted to citrulline·min\(^{-1}\)·mg protein\(^{-1}\); L-arginine transport is expressed as pmol arginine transported·min\(^{-1}\)·mg protein\(^{-1}\); and intracellular L-arginine concentration is expressed as μM calculated from L-arginine content in μmol/g total cell protein and factored for volume, assuming 1 mg intracellular protein = 6 μl intracellular water (34). HDMEC, human dermal microvascular endothelial cells; HGEC, human glomerular endothelial cells; BAEC, bovine aortic endothelial cells; NOS, nitric oxide synthase; L-NMA, N-monomethyl-L-arginine. *P < 0.05 vs. control.

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**Fig. 5.** A representative Western blot for endothelial nitric oxide synthase and β-actin on lysates of BAEC (75 μg total protein) after 7 days of treatment with either regular media (Control, lanes 2 and 5), 25 mM urea in regular media (Urea, lanes 4 and 7) or, as osmotic control, 25 mM mannitol in regular media (Mannitol, lanes 1, 3, and 6). eNOS, endothelial nitric oxide synthase.

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UREMIA INHIBITS ENDOTHELIAL L-ARGININE TRANSPORT

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into the endothelial cell, it may inhibit volume-sensitive transporters (e.g., Na\(^+\)-H\(^+\) exchanger) by “macro-molecular crowding,” leading to some unspecified inhibitory action on the pump (20). At the moment, we cannot provide a molecular mechanism for our finding, although the presence of urea transporters on cultured endothelial cells (32) suggests that urea may act intracellularly. Of note, plasma from uremic patients actually enhances L-arginine transport into red blood cells and platelets (17, 18); thus the action of urea may vary with different cell types.

Given that uremic levels of urea can inhibit the endothelial cell L-arginine transporters in vitro, what is the functional significance of this finding? We observed significant reductions of eNOS activity after 7-day (but not 6-h) incubation with 25 mM urea that is clearly not due to decreased eNOS abundance. Possibly it may take longer than 6 h for intracellular arginine levels to become sufficiently depleted that substrate availability becomes rate limiting. Although we do not usually think of substrate limitation as a mechanism for reduction of constitutive NO generation, arginine-depleted cells do not transport arginine any faster than replete cells (24); thus there is no compensatory feedback mechanism to prevent progressive falls in intracellular arginine concentration. The total intracellular arginine concentration measured in our studies is clearly not reduced by chronic exposure to urea, and calculated L-arginine concentrations are well above the \( K_m \) of eNOS (5). However, because the CAT transporters colocalize with eNOS in the endothelial cell caveolae (16), local arginine deficiency may develop in this cellular microdomain. In other words, it may be the local caveolar L-arginine concentration, determined by the CAT transporters, rather than total intracellular L-arginine concentration that determines eNOS activity, explaining the “arginine paradox” (16). Total intracellular L-arginine concentrations may reflect intracellular L-arginine synthesis, L-arginine utilization by enzymes such as arginase, and/or L-arginine efflux from the cell by other transporter systems. It is therefore possible that increased BUN may contribute to the development of hypertension in vivo, via inhibition of L-arginine transport into endothelial cells and eventual reduction in eNOS activity. In addition, high BUN may contribute to the immune dysfunction of ESRD because it is reported to inhibit NO production from activated macrophages in vitro (23). Because inducible NOS activity relies on a parallel upregulation of membrane arginine transporters (3), substrate depletion may also play a role in urea-induced inducible NOS inhibition.

The findings discussed above motivated our companion paper that investigated the impact of 7 days of uremic BUN in otherwise normal rats (32). In this paper, we report that uremic BUN has no measurable inhibitory effect on the functional systemic and renal indices of the NO system. One interpretation of this finding is that uremic blood chemistry requires elevated concentrations of multiple solutes and/or must be accompanied by other changes of renal failure, possibly reduced L-arginine synthesis by the kidney.

In summary, uremic levels of urea can inhibit L-arginine transport into cultured endothelial cells and reduce eNOS activity after 7 days of exposure. This may be functionally significant in end-stage renal disease and may contribute to the lower total NO production seen in these patients.

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