Uremic levels of BUN do not cause nitric oxide deficiency in rats with normal renal function

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Xiao, Shen, Aaron Erdely, Laszlo Wagner, and Chris Baylis. Uremic levels of BUN do not cause nitric oxide deficiency in rats with normal renal function. Am J Physiol Renal Physiol 280: F996–F1000, 2001.—In vitro, 7 days of high blood urea nitrogen (BUN) inhibits endothelial L-arginine transport and nitric oxide synthase (NOS) activity. The present study investigates whether 7 days of high BUN in vivo influences renal hemodynamics, blood pressure (BP), and/or the nitric oxide (NO) system. Normal rats were fed low-nitrate food containing 30% urea for 7 days, which increased BUN (15 ± 1 to 69 ± 4 mg/100 ml, P < 0.001). High BUN did not reduce 24-hour urinary nitrite/nitrate excretion (a measure of total NO production). Baseline BP and renal hemodynamics were unaffected by high BUN as were the pressor and renal vasoconstrictor responses to acute NOS inhibition with \( \text{N}^6 \)-nitro-L-arginine-methyl ester. In addition, high BUN had no impact on renal cortical L-arginine concentration, density of either endothelial NOS or neuronal NOS protein, or renal cortical NOS activity. NOS activity in the brain cerebellum was also unaffected. In conclusion, high BUN did not lead to vasoconstriction or NO deficiency in rats with normal renal function. Further studies are needed to evaluate the effect of high BUN on the NO system in rats with progressive renal functional insufficiency.

blood urea nitrogen; conscious rat; blood pressure; renal vascular resistance

UREA IS A WASTE PRODUCT OF protein metabolism made in the liver and excreted via the kidneys (13). When renal function is normal, blood urea nitrogen (BUN) is low, but when renal failure develops, solutes normally excreted by the kidney (including urea) accumulate in plasma to cause “uremia.” Although high BUN has classically been considered an inert marker for loss of renal function, rather than a uremic toxin, there is increasing evidence that elevated BUN can adversely affect a number of cellular processes (8). For example, uremic concentrations of urea inhibit the Na-K-2Cl cotransporter, and the activity of inducible nitric oxide synthase (NOS), as well as other enzymes (9, 12, 14, 16, 18). In addition, we found that a uremic level of urea (−75 mg/dl BUN) significantly inhibited L-arginine transport into cultured human and bovine endothelial cells after 6-h and 7-day incubation, and at 7-day NOS activity was also reduced (23). Because a similar inhibitory effect is seen with plasma from end-stage renal disease (ESRD) patients (23), we suggest that in uremia the high BUN may have a general, widespread inhibitory effect on L-arginine transport into vascular endothelial cells.

To investigate this possibility, we chronically fed normal rats a high-urea diet to produce uremic plasma concentrations of BUN. We conducted studies to measure baseline blood pressure and renal function in conscious rats at normal BUN and after 7 days of high BUN, when the activity of the nitric oxide (NO) system was also acutely tested by systemic NOS inhibition. We also measured the impact of high BUN on total NO production from 24-h urinary excretion of NO\(_2^+\) + NO\(_3^-\) [nitrite/nitrate (NOx); the stable oxidation products of NO], NOS activity in renal cortex and brain cerebellum, and the abundance of NOS proteins in the kidney.

METHODS

Studies were performed on 14 male Sprague-Dawley rats (3–5 mo) obtained from Harlan Sprague Dawley, (Indianapolis, IN). Six of the rats were chronically implanted with bladder and vascular catheters as described by us previously (1–3, 17, 22). Rats were maintained on low-nitrate food (ICN AIN76C semipurified diet, ICN Pharmaceuticals, Costa Mesa, CA; NOx content 125 μmol/kg) throughout the study. After a control 2-h urine collection, rats were catheterized under general anesthesia using fully sterile techniques. At least 7 days later, after recovery, a clearance experiment was conducted to measure blood pressure and renal function in the control (normal BUN) state. Rats then received the low-nitrate food supplemented with 30% urea for the remainder of the study. Rats were given 20 g urea supplemented food at 12-h intervals for 1 wk, all of which was consumed (equivalent to 5 μmol NOx ingested in 24 h). This is an adaptation of a protocol in which urea feeding leads to elevated BUN in rats with normal renal function (14). A 24-h urine collection was made at days 5–6 on urea-supplemented rats, and after a 24-h recovery a final renal function experiment was conducted in which baseline blood pressure (BP) and renal...
function (high BUN) measurements were made, followed by acute, systemic NOS inhibition (see below); the rats were then euthanized, and the bladder and kidneys were inspected to ensure absence of infection.

The following measurements were made in the renal function experiments: BP, glomerular filtration rate (GFR), and renal plasma flow (RPF) by measuring the clearance of insulin and p-aminohippuric acid (PAH), respectively, and sodium excretion. Fractionation (FF) and renal vascular resistance (RVR) were calculated. After completion of control measurements (average of 2 clearance periods) at normal BUN, each rat was returned to its home cage and the urea-supplemented diet began. Seven days later, renal function measurements were repeated in the baseline state (at high BUN) and then during acute systemic inhibition of NOS (N\textsuperscript{\text{N}}-nitro-L-arginine methyl ester (L-NAME), 10 mg/kg iv; a supramaximal pressor dose in this preparation (3)). Details of the experimental protocols, analytic techniques, and calculations have been published by us previously (1–2, 17, 22).

In addition, the NOx levels were measured in 24-h urine samples, as described previously (22). Urinary protein concentration was determined by the Bradford assay (5), and BUN levels were measured by Sigma kit no. 640.

An additional group of four rats was fed an identical high-urea diet for 7 days, and then tissues were harvested, snap-frozen, stored at \(-80^\circ\text{C}\) for later in vitro study, and compared with tissues from four control rats fed on a normal low-NOx diet. The following measurements were made: arginine concentration was measured in homogenates (in 0.9% NaCl) from aorta and kidney cortex, using a reverse-phase HPLC method described by us previously, except that the column is run at 41°C (20). NOS activity was measured from the conversion of \([3\text{H}]\)arginine to \([3\text{H}]\)citrulline in cerebellum and renal cortex as follows. Frozen tissue was homogenized in a volume of chilled homogenization buffer (15), five times the tissues’ wet weight (in g). The crude homogenate was sonicated for 1 min and then centrifuged at 100,000 \(g\) for 60 min at 4°C to obtain soluble and membrane fractions (6). Endogenous arginine was removed as follows: the soluble fraction was combined 1:1 with Dowex resin (sodium form), vortexed, and centrifuged at 1,370 \(g\) for 3 min at 4°C; the membrane pellet was resuspended in 5 ml of homogenization buffer and centrifuged at 100,000 \(g\) for 60 min at 4°C, and the resulting pellet was resuspended in 1 ml of homogenization buffer to obtain a membrane fraction with endogenous arginine removed (21). NOS activity was determined by measuring the conversion of \([3\text{H}]\)arginine to \([3\text{H}]\)citrulline (6) with minor modifications. Briefly, an aliquot of soluble or membrane fraction was mixed with assay buffer; final concentrations were 25 mM Tris-HCl, 1.25 mM CaCl, 1 mM NADPH, 15 \(\mu\text{M}\) \([3\text{H}]\)arginine, 5 \(\mu\text{M}\) L-arginine, 1.09 \(\mu\text{M}\) Flavin adenine dinucleotide, 1.09 \(\mu\text{M}\) Flavin mononucleotide, 0.33 mM tetrahydrobiopterin, 0.11 \(\mu\text{M}\) calmodulin, and 1.09 \(\mu\text{M}\) dithiothreitol. The arginase inhibitors, 10 mM valine and 10 mM proline, were added to the renal cortex assay. For each sample, two sets of triplicates were run, one at baseline and one in the presence of the nonspecific NOS inhibitors N\textsuperscript{\text{N}}-methyl-L-arginine (L-NMA) (10 mM) and L-NAME (20 mM) for kidney, and N\textsuperscript{\text{N}}-nitro-L-arginine (L-NNA; 2 mM) for the cerebellar fractions. The inhibitors were incubated at room temperature for 15 min to allow sufficient interaction with the NOS enzyme, then L-arginine was added, and samples were incubated for 45 min in a 37°C shaking water bath. The reaction was stopped with 400 \(\mu\text{l}\) of chilled stop buffer (5 mM EDTA and 50 mM HEPES at pH 5.5), and then 400 \(\mu\text{l}\) of 50:50 AG50W-X8 Dowex resin (sodium form) were added to each sample tube. Samples were vortexed (5 min), centrifuged at 3,100 \(g\) for 3 min, and then 500 \(\mu\text{l}\) of the supernatant were added to 4 ml OptiFluor scintillant and counted on a Packard scintillation counter. Data are expressed as pmoles of L-[\(3\text{H}\)]citrulline converted per minute per milligram protein (pmol citrulline·min\(^{-1}\)·mg protein\(^{-1}\)) and corrected for background. Background was determined on heat-inactivated samples (incubated for 60 min at 80°C). The background standard was prepared by removing the nonconverted L-[\(3\text{H}\)]arginine with the Dowex resin; this background value is <5% of the 100% standard, representing free tritium or noncaticonic tritiated species not bound by the Dowex resin. The 100% standard was prepared by adding 200 \(\mu\text{l}\) of water instead of the Dowex; this value represented the total amount of counts available for conversion.

The abundance of endothelial (eNOS), neuronal (nNOS), and \(\beta\)-actin proteins was measured on homogenates of kidney cortex as follows. Frozen tissue was homogenized in lysis buffer (1 mg/5 \(\mu\text{l}\) at 4°C, and centrifuged at 10,000 \(g\) for 10 min at 4°C. Proteins from the supernate were electrophoretically separated on SDS-PAGE at 200 V for 65 min at 4°C. Sample wells were loaded (100 \(\mu\text{g}\) of protein at constant volume; 50 \(\mu\text{l}\)) and compared with high-molecular-weight range prestained (blue) standards (Bio-Rad). Proteins were transferred from the gel to nitrocellulose membranes in a Bio-Rad transblot SD semidyey transfer cell for 1 h 45 min at 60 mA. eNOS was detected (after 1-h blocking) by incubation with mouse monoclonal antibody (Transduction Laboratories) against eNOS (1:250 dilution for 1 h, room temperature), then incubated with secondary antibody [goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate, 1:2,000 at room temperature for 1 h, Transduction Laboratories]. Membranes were then incubated with enhanced chemiluminescence (Amersham) and exposed to Kodak X-OMAT AR film. Membranes were stripped (11), and for detection of nNOS the membrane was incubated with rabbit polyclonal antibody against nNOS (raised against the NH\textsubscript{2} terminus (231 amino acids) of rat nNOS; gift from Dr. Kim Lau, UT Dallas), then incubated with secondary antibody [goat anti-rabbit, in goat IgG-HRP conjugate (Bio-Rad), 1:5,000 dilution for 1 h at room temperature]. Membranes were again stripped and reprobed with \(\beta\)-actin (Sigma mouse antibody, A6441, 1:60,000 dilution for 1 h at room temperature; secondary antibody; anti-mouse in goat IgG-HRP, 1:60,000 dilution for 1 h at room temperature). For additional details, see Mattson and Bellehumeur (11). eNOS and nNOS abundance was measured by image analysis (Optimas 6.2, Bothell, WA) of the integrated optical density, normalized for \(\beta\)-actin. Results are expressed as means ± SE. Statistical analysis of the functional data was by paired \(t\)-test, between the pre- and posttreatment controls (normal and high BUN) and baseline vs. acute NOS inhibition during high BUN. Unpaired \(t\)-testing was used for statistical analysis of the in vitro measures. Values of \(P < 0.05\) are considered to be significantly different.

RESULTS

As shown in Fig. 1, BUN increases to a stable, high value by day 3 of urea supplementation. After 7 days of urea feeding, BUN was \(\sim 4 \times\) normal control value (Table 1). We measured 24-h urinary excretion of NO\textsubscript{2} + NO\textsubscript{3} (UNO\textsubscript{X}V) as an index of total body NO production and, contrary to our hypothesis, we saw no fall in UNO\textsubscript{X}V after 7 days of high BUN; in fact, UNO\textsubscript{X}V increased moderately (Table 1). Dietary NOx intake
was constant at 5 μmol/24 h, and because only 50–70% of ingested NOx enters the body and NOx output in the urine always exceeded intake, total NO production must have increased as a result of the urea feeding. The 24-h urinary protein excretion also increased (Table 1).

A sustained rise in BUN for 1 wk did not affect the BP, RVR, RPF, or GFR (Table 1). However, the urine osmolarity was markedly elevated, whereas urinary excretion of Na fell. As shown in Fig. 2, acute systemic administration of the NOS inhibitor L-NAME produced a significant increase in BP in rats with elevated BUN, as well as a marked rise in RVR (a fall in RPF, not shown) with a more modest reduction in GFR. This is a similar pattern of hemodynamic responses seen in normal control rats studied in this same preparation and reported by us previously (2, 3, 17). The only difference in the response to L-NAME in rats with high BUN was that the increase in urinary sodium excretion observed in normal control rats (2, 3, 17), was absent (Fig. 2).

As shown in Table 2, the L-arginine concentration in the kidney cortex was similar in control and urea-fed rats. The abundance of eNOS and nNOS protein (relative to β-actin) in the renal cortex was similar in the normal and high-BUN rats. A representative blot for eNOS is given in Fig. 3. Furthermore, the NOS activity of the soluble fraction of the renal cortex was similar in the two groups (Table 2). Because the conversion of L-arginine to L-citrulline was ~90% inhibited in the presence of L-NMA + L-NAME, this activity is due to the NOS enzyme in both groups. We also measured the NOS activity in the cerebellum (a high-output site relative to the kidney), which was similar in both soluble and membrane fractions (29 ± 7 vs. 35 ± 4 and 13 ± 2 vs. 11 ± 1 pmol citrulline·min⁻¹·mg protein⁻¹, respectively) with similar inhibition in the presence of the NOS inhibitor L-NNA, which averaged 99.2 ± 0.3%.

DISCUSSION

The main finding in this study is that when renal function is normal, a “uremic” level of BUN maintained for 7 days causes no adverse changes in BP, renal hemodynamics, or the NO system. The motivation for the present study was our in vitro observation that after 7-day incubation, both L-arginine transport and eNOS activity are inhibited in cultured endothelial cells grown in a high-urea environment (23). We had

Table 1. Summary of measurements in conscious control rats and rats after 7 days of dietary urea feeding, made in the baseline state

<table>
<thead>
<tr>
<th></th>
<th>UNoxV, μmol/24 h</th>
<th>UmV, mg/24 h</th>
<th>BUN, mg/100 ml</th>
<th>BP, mmHg</th>
<th>RVR, mmHg·ml⁻¹·min⁻¹</th>
<th>RPF, ml/min</th>
<th>GFR, ml/min</th>
<th>Uosm, mosm/l</th>
<th>UNaV, μmol/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.9 ± 1.1</td>
<td>37 ± 11</td>
<td>15 ± 1</td>
<td>118 ± 3</td>
<td>8.4 ± 1.6</td>
<td>9 ± 1</td>
<td>2.3 ± 0.2</td>
<td>604 ± 75</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>+ Urea</td>
<td>10.4 ± 0.7</td>
<td>88 ± 15</td>
<td>69 ± 4</td>
<td>123 ± 6</td>
<td>6.8 ± 1.6</td>
<td>11 ± 1</td>
<td>2.5 ± 0.2</td>
<td>1198 ± 83</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 rats. Urinary NO₂ + NO₃ (NOx) excretion (UNoxV) and total protein excretion (UmV) are from metabolic cage studies. Blood urea nitrogen values (BUN), mean arterial blood pressure (BP), renal vascular resistance (RVR), renal plasma flow (RPF), glomerular filtration rate (GFR), urinary sodium excretion (UNaV), and urine osmolality (Uosm) were measured during the acute renal function studies. NS, not significant.
might eventually cause intracellular L-arginine deficiency and thus inhibit endothelial NO production in vivo.

Urea is a breakdown product of L-arginine formed mainly in the liver by the action of arginase (13). The synthesis of L-arginine and its subsequent conversion to urea is a major metabolic pathway that serves to eliminate nonessential nitrogen-containing compounds from the body. The urea, once formed, is not further metabolized and is distributed in the total body water and excreted by the kidney (19). In health and under conditions of relatively constant intake of protein, the plasma and urine levels of urea may serve as an index of renal function, dietary protein intake, and hydration in the adult mammal.

In ESRD, the plasma concentration of urea increases 5-10× above control, due to loss of renal clearance (20). Although previously regarded as an innocuous uremic metabolite, recent evidence indicates that urea interferes with cellular metabolism and protein synthesis, transport of electrolytes, and also synthesis of a number of enzymes (8, 9, 14, 18). Of note, it has been reported that high urea levels inhibit inducible NOS activity and NO production from stimulated macrophages (12, 16), an effect that may account for some of the immune suppression of advanced renal failure. This evidence, together with our findings that NO production is reduced in ESRD patients (20) and that high urea levels inhibit L-arginine transport into endothelial cells (23), led us to investigate the impact of high BUN in the absence of renal damage.

Table 2. Summary of in vitro measurements in kidney cortex of control rats and rats given urea-supplemented food for 7 days

<table>
<thead>
<tr>
<th></th>
<th>eNOS/β-Actin, IOD</th>
<th>nNOS/β-Actin, IOD</th>
<th>NOS Activity pmol citrulline·min⁻¹·mg protein⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.3 ± 0.6</td>
<td>0.3 ± 0.1</td>
<td>0.82 ± 0.13 (89 ± 1)</td>
</tr>
<tr>
<td>+ Urea</td>
<td>13.1 ± 0.5</td>
<td>0.2 ± 0.1</td>
<td>0.84 ± 0.11 (86 ± 2)</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 rats in each group. Values in parentheses give % inhibition of nitric oxide synthase (NOS) activity in the presence of NOS inhibitors. The tissue L-arginine concentration [L-arg], the endothelial and neuronal NOS (eNOS and nNOS, respectively) protein abundance (relative to β-actin) in integrated optical density (IOD) units and NOS activity in the soluble fraction are shown.

To test the direct effect of urea on the L-arginine-NO system, we designed experiments in vivo to evaluate the effects of elevated BUN on NO production (by measuring the 24-h NOx production = NOx excretion), blood pressure, and renal function. To avoid interference by NOx ingested in the diet, which confounds interpretation of UNOxV data (4), rats were fed with low-nitrate food. Because of this precaution, we think it likely that the 24-h UNOxV reported here gives a valid, qualitative index of the total NO production. Contrary to our hypothesis, 24-h UNOxV did not fall as a result of sustained high BUN, suggesting that total NO production (from all tissue sources) is not impaired by a uremic blood level of urea per se. In fact, there was a small rise in 24-h UNOxV (24-h urinary protein excretion) with high BUN. We are reluctant to conclude that this means increased total NO production with urea feeding, however, in view of the major renal tubular effects of such a large urea load. Because these animals with high BUN had normal renal function, they were undergoing a profuse osmotic diuresis during the 7 days of urea feeding. Both NOx and filtered plasma proteins normally undergo significant proximal reabsorption (22), and it is likely that the increases in 24-h urinary protein and NOx excretions reflect a “washout” effect secondary to the proximally acting osmotic diuretic action of excess urea. The only difference in the response to acute NOS inhibition seen in rats with high BUN (discussed below) was that the increase in urinary sodium excretion observed in normal control rats (2, 3, 17) was totally blocked by the high uremic level of BUN, because urine flow was already markedly elevated due to the urea feeding. We presume that this reflects another facet of the osmotic diuretic effect of high BUN in animals with normal renal function.

The 24-h UNOxV reflects total NO production, and because the NO from eNOS is likely to be a small fraction of this total, UNOxV may have little predictive power of directional changes in eNOS activity. We also measured BP because NO generation from eNOS provides a powerful tonic vasodilatory stimulus (2, 10). In our conscious, chronically catheterized rat preparation, high BUN for 7 days did not affect the baseline BP or renal hemodynamics. Furthermore, the pressor response to acute NOS inhibition in the high-BUN state was robust and similar to that seen in several earlier studies by us in rats with normal BUN (2, 3, 17). These functional findings, together with our in vitro observa-

Fig. 3. Representative Western blot for endothelial NOS (eNOS), showing positive control (75 µg bovine endothelial cell lysate) and 100 µg total protein from kidney cortex in 2 normally fed rats and 3 urea-fed rats. The same blot was also later probed with β-actin and shows equal loading per lane.
tions that renal arginine production, NOS activity, and protein abundance are unaffected by 7 days of high BUN, suggest that additional factors are necessary in vivo before the NO system is impaired.

In conclusion, in contrast to our in vitro studies (23) we found that in vivo 7 days of high BUN has no perceptible NOS inhibitory action. These in vivo studies were conducted in normal rats with functional kidneys. Thus endogenous NOS inhibitors are excreted and L-arginine is synthesized optimally by the native kidneys. We suggest that when arginine availability is compromised and the clearance of endogenous NOS inhibitors is reduced (as in renal failure), the transport inhibitory action of urea on L-arginine may combine with these other effects to decrease NO production.

We are grateful to Kevin Engels and Lennie Samsell for excellent technical assistance. The nNOS antibody was kindly supplied by Dr. Kim Lau (Dept. of Physiology, UT Dallas).

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REFERENCES