Chronic L-arginine administration increases oxidative and nitrosative stress in rat hyperoxaluric kidneys and excessive crystal deposition

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Huang H-S, Ma M-C, Chen J. Chronic L-arginine administration increases oxidative and nitrosative stress in rat hyperoxaluric kidneys and excessive crystal deposition. Am J Physiol Renal Physiol 295: F388–F396, 2008. First published April 30, 2008; doi:10.1152/ajprenal.00405.2007.—Hyperoxaluric kidneys show an impaired diuretic response to acute infusion of L-arginine. In this study, we examined the chronic effect of L-arginine supplementation on CaOx crystal formation in hyperoxaluric rat kidneys. Eight groups were tested: control (received drinking water), L group (received L-arginine, 0.6%), LN group [received Nω-nitro-L-arginine methyl ester (L-NAME), HP group [received hydroxy-L-proline (HP, 5%) mixed with chow to induce hyperoxaluria], L + HP group (received HP + L-arginine), HP + LN group, and L + HP + LN group. The duration was 42 days, and each group had eight animals. Urinary biochemistry and renal CaOx amounts were measured, as well as renal expressions of nitric oxide synthase (NOS) isoforms and NAD(P)H oxidase. The distribution of inducible NOS (iNOS), NAD(P)H oxidase, ED1-positive cells, and nitrotyrosine was examined by immunohistochemical and immunofluorescence studies, whereas superoxide production from the kidneys was examined by fluorescence spectrometric assay. Compared with the HP group, the L + HP group had excessive CaOx crystal accumulation and enhanced endothelial NOS (eNOS), iNOS, and NAD(P)H oxidase protein expression in the kidney. Urinary excretion of nitrosative stress was markedly increased. Increased superoxide formation in the L + HP kidney was derived from NAD(P)H oxidase and uncoupled eNOS, and increased nitrotyrosine formation might derive from iNOS and ED1-positive cells that gathered around the CaOx crystals. L-NAME cotreatment (L + HP + LN group) reduced renal oxidative and nitrosative stress and tubular damage, which were induced by L + HP. The results showed that chronic L-arginine treatment to the hyperoxaluric kidney with massive CaOx crystal deposition may have a toxic effect by enhancing intrarenal oxidative and nitrosative stress.

hyperoxaluria; nephrolithiasis; NAD(P)H oxidase; nitric oxide synthase

HYPEROXALURIA IS THE RESULT of either genetic or environmental factors that can lead to urolithiasis, nephrocalcinosis, metabolic acidosis, hematuria, and renal failure (19). Because there are many similarities between experimental nephrolithiasis and human kidney stone formation, the rat model of calcium oxalate (CaOx) nephrolithiasis can be used to investigate mechanisms involved in human kidney stone formation (13–16).

Nitric oxide (NO) plays a critical role in multiple renal processes, including the regulation of renal plasma flow, glomerular filtration rate, generation of renin and ANG II, and tubular reabsorption (5). Endogenous NO is synthesized by nitric oxide synthase (NOS) using L-arginine (L-Arg) as a substrate. Three isoforms of NOS, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), are expressed in the kidneys (1, 7). Evidence has been accumulating that, in pathological states, iNOS may be overexpressed, and both eNOS and nNOS expression can be altered (1). It has been reported that L-Arg infusion in the kidneys can stimulate intrarenal NO production by enhancing NOS activity in vivo (8, 38). Intrarenal NO production stimulated by L-Arg also increases renal sodium excretion by a direct tubular action and by regulating vascular tone in the pressure natriuretic response in the renal medulla (2, 5, 8). However, the hyperoxaluric kidney showed a poor renal excretion when responding to acute saline loading (activates endogenous NO system) or exogenous L-Arg infusion, and this was associated with less urinary NO metabolites and cGMP excretion (17). These suggested that the hyperoxaluric kidney may not properly respond to the acute increased NO formation.

Our previous study showed that CaOx crystal deposition causes a change in the balance of antioxidant-oxidant, and macrophage infiltration in the kidney may contribute to this redox imbalance (13). Nitrosative stress is also induced in kidneys with renal stones (17, 29), and the production of reactive nitrogen species may cause tubular damage and exacerbate the disease.

The symptoms in many models of experimentally induced renal disease, such as cyclosporine-induced nephropathy, diabetic nephropathy, and ureteral obstruction, are improved by chronic dietary supplementation with L-Arg (11, 31). The administration of L-Arg to injured kidneys improves excretory response and is reported to reduce lipid peroxidation (LPO) in patients with diabetes mellitus (24). Aside from its beneficial effects, excessive NO has several cytotoxic effects, including peroxynitrite formation, NO-induced nitrosylation, and the inactivation of various enzymes (7). Therefore, we aimed to study the effects of L-Arg on CaOx crystal formation after hyperoxaluria. Because hydroxy-L-proline (HP), a physiologically precursor of oxalate, has been reported to induce hyperoxaluria and CaOx crystal formation but without an associated metabolic acidosis when mixed in the diet at 5% (19), we therefore conducted the HP-induced hyperoxaluric model. Our present study unexpectedly showed that L-Arg caused oxidative and nitrosative stress in the hyperoxaluric kidney; this favored more crystal formation. We therefore explored the...
blockade effect of NOS by $N^2$-nitro-$L$-arginine methyl ester ($L$-NAME) to see whether NOS inhibition is beneficial to the hyperoxaluric kidney.

**METHODS**

*Animals.* Male Wistar rats (180–220 g) were housed at a constant temperature and on a light-dark cycle (light from 0700 to 1800). Animal care and experimental protocols followed the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington DC, 1997) and were reviewed and approved by the Institutional Animal Care and Use Committee, National Taiwan University School of Medicine.

The rats were divided into eight groups that underwent treatment for 42 days. The control group received drinking water, the $L$-NAME group received 0.6% $L$-Arg (Sigma, St. Louis, MO) (6) in drinking water, and the $L$ + $H$ group received 0.6% $L$-Arg and 0.6% $L$-NAME. The last four groups were given chow mixed with 5% HP (wt/wt HP/chow) purchased from ICN Biochemicals (Aurora, OH) (19). The HP group received only this treatment, the $L$ + HP group received HP and 0.6% $L$-Arg in the drinking water, the $L$ + HP + $L$-NAME group received HP with 0.6% $L$-Arg and $L$-NAME in the drinking water, and the $H$ + $L$ group received HP and $L$-NAME. The drugs were used at doses indicated as effective in the literature (1, 6, 19) and which prevented toxic activity. There were eight rats ($n = 8$) in each group.

During the experimental period, all groups had free access to standard rat chow. The animals were placed in metabolic cages 3 days before surgery for acclimatization.

*Biochemical assays.* Twenty-four-hour urine samples were collected 1 day before death and were analyzed using kits for oxalate (Sigma), citrate (R-Biopharm, Darmstadt, Germany), N-acetyl-$\beta$-glucosaminidase (tubular damage marker) (13), malondialdehyde (MDA), LPO marker (kit from Oxis Research, Portland, OR), NO metabolites (nitrate and nitrite were purchased from Cayman Chemical, Ann Arbor, MI), and cGMP (Assay Designs Ann Arbor, MI), which had been used as markers of systemic NO production, and bioactivity (17). All samples were assayed in duplicate.

Creatinine (serum and urine), urine electrolytes (calcium and magnesium), and total urine protein were measured at the Central Laboratory of the National Taiwan University Hospital. Urinary supersaturation with respect to CaOx was assessed using the index proposed previously (35). Sediments in the 24-h urine sample were collected and examined for the presence of CaOx crystals by polarized microscopy, as described below. These were then weighed, placed in an oven for 48–72 h, and weighed daily until a stable weight was achieved.

Myeloperoxidase (MPO) levels, an indicator of tissue neutrophil accumulation (1) in the kidneys, were measured colorimetrically (Assay Designs).

Urinary nitrotyrosine (NTS) level, an indicator of peroxynitrite formation, was also measured colorimetrically (Kamiya Biomedical, Seattle, WA). The presence of CaOx crystals in the kidneys was examined and graded as described previously (13). Each kidney was scored semiquantitatively as one of four grades (0, I, II, and III), ranging from “no crystal deposit” to “massive crystal deposits.” Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels, oxidative stress markers, in the kidneys were measured colorimetrically (Oxis Research), and the GSH redox ratio was calculated using the following equation: 
\[
\text{redox ratio} (\%) = \frac{2 \times \text{GSSG}}{\text{GSH} + 2 \times \text{GSSG}} 
\]

*Luminol-dependent chemiluminescence assay.* We compared chemiluminescence (CL) counts on the kidney surface in the different groups. Details of the procedures are described in our earlier publication (13). CL was expressed as a counts per 10 s.

**Fluorescence spectrometric assay of O$_2^-$ production from the kidneys.** Fluorogenic oxidation of dihydroethidium to ethidium was used as a measure of O$_2^-$ (10). The isolated kidneys were homogenized in ice-cold buffer. After centrifugation at 6,000 g for 5 min at 4°C, the supernatant containing the membrane and cytosolic components was separated. The homogenates (20 μg) were then incubated at 37°C for 30 min with dihydroethidium (0.02 mM; Sigma-Aldrich Japan), salmon testes DNA (0.5 mg/ml), and various substrates for superoxide-producing enzymes and their inhibitors in a microtiter plate placed away from direct light.

Ethidium-DNA fluorescence was measured at an excitation of 480 nm and an emission of 610 nm using a fluorescence microplate reader (Perkin-Elmer, Boston, MA; NADH (0.1 mM) was used as a substrate for NAD(P)H oxidase. Diphenylene iodonium chloride (DPI, 0.1 mM) was then added to inhibit NAD(P)H oxidase. The effect of BH$_4$ (0.01 mM) on $L$-arginine-induced O$_2^-$ production was examined.

**Immunohistochemical staining.** Details of the procedures are described in our earlier publication (14). The paraffin-embedded tissue sections were incubated for 1 h at room temperature (RT) with mouse monoclonal antibody against NTS (1:100 dilution; Cayman Chemical) or against the macrophage/monocyte antigen ED1 (1:1,000 dilution; Serotec, Oxford, UK) (13). Bound primary antibody was detected using an ABC kit with anti-mouse IgG antibody and 3,3'-diamino-benzidine as substrate. Gill’s hematoxylin was used as counterstain.

The method for calculating the number of ED1-positive cells in these sections has been previously described (13).

**Immunofluorescence examination of iNOS and gp91phox (NAD(P)H oxidase).** Rats were anaesthetized and then perfused transcardially with saline. The presence of CaOx crystals in the kidneys was examined. The sections were then rehydrated and washed with PBS, the sections were incubated overnight at 4°C with rabbit anti-iNOS antibody (Santa Cruz) or against the macrophage/monocyte antigen ED1 (1:1,000 dilution; Serotec, Oxford, UK) (13). Bound primary antibody was detected using an ABC kit with anti-mouse IgG antibody and 3,3'-diaminobenzidine as substrate. Gill’s hematoxylin was used as counterstain.

After being rehydrated and washed with PBS, the sections were processed for indirect immunofluorescence. After being blocked with 5% skim milk in PBS for 1 h at RT, the sections were incubated overnight at 4°C with rabbit anti-iNOS antibody (Santa Cruz) or mouse anti-gp91phox antibody (Santa Cruz) diluted 500-fold in 5% normal rabbit serum in PBS or 400-fold in PBS, respectively, and then incubated for 1 h at RT with rhodamine-Red-X-conjugated donkey anti-rabbit antibody (1:100 in 5% skim milk; Jackson Immuno Research, West Grove, PA) for iNOS, or conjugated with fluorescein isothiocyanate.

Tissue sections were examined on an Olympus BX51 microscope (Tokyo, Japan) with a fluorescent image analytic system (Diagnostic Instruments) at ×200 magnification. Nuclei were counterstained by 4',6'-diamidino-2-phenylindole. The specificity of the antibody was tested by preincubation of the primary antibody with blocking peptide (Santa Cruz).

**Western blot.** Details of the procedures of Western blot analysis are described in our earlier publication (17). In brief, the same amount of protein from each preparation was loaded on each lane and nitriloacetaic membrane (Amersham Bioscience, Buckingham, UK) and then incubated overnight at 4°C with monoclonal mouse antibodies against eNOS (1:500), iNOS (1:250), nNOS (1:500), NTS (1:250), poly- (ADP-ribose) polymerase (PARP, 1:250) (all from BD Biosciences), gp91phox (1:2,000) (Santa Cruz Biotechnology, Santa Cruz, CA), or actin (1:1,000; Sigma) (Cedarlane, Ontario, Canada) in the blocking solution.

The density of the band in each lane was determined semiquantitatively using a densitometer with an image analytic system (Diagnostic Instruments) and expressed as the ratio of the density units for the band divided by those for actin.
RESULTS

Body data and urinary biochemistry. Supplementation with 0.6% l-Arg resulted in a 2.26-fold higher daily intake of l-Arg (560 ± 38 mg/day in the L group vs. 248 ± 13 mg/day in the controls) (Table 1). Food consumption (data not shown) and body weight (Table 1) did not differ among the groups. Water intake was significantly increased in all l-treated groups, whereas kidney weight was significantly increased in all HP-treated groups (Table 1). Creatinine clearance did not differ among the groups.

Hyperoxaluria was seen in all HP-treated groups and was associated with significantly increased excretion of tubular markers. However, increments in the L + HP + LN group were significantly lower than those of the L + HP group. l-Arg supplementation resulted in a significant increase in urinary NOx and cGMP levels in the L and LP groups, which returned to normal levels after cotreatment with l-NAME (L + LN and L + HP + LN groups). Changes in the urinary pH value in the experimental groups had no statistical significance when compared with the controls (data not shown).

Compared with the control group, urinary excretion of NTS increased significantly in all HP-treated groups and was significantly higher in the L + HP group than in the HP and L + HP + LN groups (Table 1). The degree of urine CaOx supersaturation was significantly higher in all HP-treated groups than in the other groups (Table 1).

l-Arg enhanced CaOx crystal deposition. In Table 1 and Fig. 1, no obvious CaOx crystal deposition was seen in the control and L, LN, and L + LN groups (data not shown). However, massive accumulation of CaOx (grade III) (Table 1) associated with a dilated tubular structure was seen in the L group (Fig. 1c) compared with the grade II seen in the HP group (Fig. 1a) and the grades I-II seen in the HP group.

Table 1. Body data and urinary biochemistry of the eight study groups

<table>
<thead>
<tr>
<th>Group Parameters</th>
<th>Control</th>
<th>L</th>
<th>LN</th>
<th>L + LN</th>
<th>HP</th>
<th>HP + LN</th>
<th>L + HP</th>
<th>L + HP + LN</th>
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<tbody>
<tr>
<td>Body wt, g</td>
<td>389.2±13.7</td>
<td>402.9±14.2</td>
<td>361.1±14.8</td>
<td>395.5±11.4</td>
<td>371.2±9.9</td>
<td>362.7±10.5</td>
<td>349.4±16.4</td>
<td>367.0±22.4</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>28.7±8.2</td>
<td>51.9±8.7*</td>
<td>38.3±8.9</td>
<td>53.2±7.1*</td>
<td>48.0±8.2</td>
<td>46.2±9.5</td>
<td>63.6±10.3**</td>
<td>52.0±4.7†</td>
</tr>
<tr>
<td>Kidney wt, g</td>
<td>2.14±0.10</td>
<td>2.05±0.10</td>
<td>2.12±0.10</td>
<td>2.11±0.10</td>
<td>2.55±0.14*</td>
<td>2.47±0.13*</td>
<td>2.69±0.13*</td>
<td>2.56±0.13*</td>
</tr>
<tr>
<td>Ccr, ml/min</td>
<td>2.0±0.3</td>
<td>1.8±0.4</td>
<td>2.0±0.4</td>
<td>2.1±0.4</td>
<td>1.7±0.2</td>
<td>1.9±0.3</td>
<td>1.8±0.2</td>
<td>2.0±0.5</td>
</tr>
<tr>
<td>MABP, mmHg</td>
<td>126.0±6.1</td>
<td>120.1±3.7</td>
<td>127.3±6.5</td>
<td>127.8±6.7</td>
<td>123.7±5.7</td>
<td>126.1±5.9</td>
<td>124.2±5.8</td>
<td>125.9±5.8</td>
</tr>
<tr>
<td>Urine output, ml/24 h</td>
<td>32.8±7.9</td>
<td>43.3±6.5*</td>
<td>31.5±6.2</td>
<td>35.1±7.2</td>
<td>32.8±8.7</td>
<td>33.3±4.8</td>
<td>44.1±6.6*</td>
<td>33.7±3.6</td>
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<tr>
<td>Urine oxalate, µmol/mg Cr</td>
<td>4.1±2.4</td>
<td>4.2±0.7</td>
<td>4.6±2.1</td>
<td>4.1±2.3</td>
<td>32.5±11.1*</td>
<td>36.8±9.4*</td>
<td>31.5±4.9*</td>
<td>28.8±2.4*</td>
</tr>
<tr>
<td>Urine citrate, mg/mg Cr</td>
<td>2.2±0.3</td>
<td>2.0±0.5</td>
<td>1.3±0.5</td>
<td>1.3±0.4</td>
<td>1.9±0.9</td>
<td>1.6±0.8</td>
<td>0.8±0.4</td>
<td>2.8±0.9</td>
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<tr>
<td>Urine Mg, µmol/24 h</td>
<td>29.2±11.9</td>
<td>31.0±9.6</td>
<td>28.7±10.3</td>
<td>29.6±11.4</td>
<td>4.0±0.9*</td>
<td>5.8±2.7*</td>
<td>6.3±3.0*</td>
<td>4.2±0.8*</td>
</tr>
<tr>
<td>Urine Ca, mg/24 h</td>
<td>0.63±0.07</td>
<td>0.83±0.19</td>
<td>0.75±0.12</td>
<td>0.58±0.05</td>
<td>0.16±0.09*</td>
<td>0.18±0.11*</td>
<td>0.33±0.18*</td>
<td>0.21±0.05*</td>
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<tr>
<td>Urinary NAG, µmol/mg Cr</td>
<td>1.9±0.4</td>
<td>3.5±6.0</td>
<td>2.5±5.0</td>
<td>2.6±0.5</td>
<td>6.3±4.9*</td>
<td>5.9±1.4*</td>
<td>13.5±0.9†</td>
<td>6.6±1.6‡</td>
</tr>
<tr>
<td>Urine NOx, nmol/24 h</td>
<td>1.85±0.31</td>
<td>2.75±0.32*</td>
<td>1.91±0.45</td>
<td>1.74±0.33</td>
<td>1.13±0.43</td>
<td>1.14±0.52</td>
<td>2.41±0.34*</td>
<td>0.91±0.33‡</td>
</tr>
<tr>
<td>Urine cGMP, nmol/24 h</td>
<td>0.7±0.1</td>
<td>1.5±0.2*</td>
<td>0.8±0.1</td>
<td>0.5±0.1</td>
<td>0.6±0.1</td>
<td>0.6±0.1</td>
<td>1.6±0.1*</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>Urine NTS, mol/g Cr</td>
<td>0.13±0.04</td>
<td>0.24±0.09</td>
<td>0.13±0.04</td>
<td>0.12±0.03</td>
<td>0.37±12.0*</td>
<td>0.36±0.04*</td>
<td>0.57±0.07*</td>
<td>0.32±0.15*</td>
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<tr>
<td>AP (CaOx)index</td>
<td>3.9±3.3</td>
<td>5.7±1.7</td>
<td>4.1±2.4</td>
<td>4.1±1.8</td>
<td>13.1±5.2*</td>
<td>11.5±4.6*</td>
<td>11.6±3.1*</td>
<td>11.3±4.5*</td>
</tr>
<tr>
<td>Dry weight of urine sediments, g</td>
<td>0.15±0.02</td>
<td>0.18±0.02</td>
<td>0.16±0.02</td>
<td>0.15±0.02</td>
<td>0.38±0.02*</td>
<td>0.42±0.02*</td>
<td>0.36±0.01*</td>
<td>0.38±0.02*</td>
</tr>
<tr>
<td>Renal score</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>II</td>
<td>I-II</td>
<td>III</td>
<td>II</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 8 rats in each group. Ccr, creatinine clearance; HP, hydroxy-L-proline; L, l-arginine; LN, Nω-nitro-L-arginine methyl ester (l-NAME); NAG, N-acetyl-β-glucosaminidase; NTS, nitrotyrosine; MABP, mean arterial blood pressure; Ca, calcium; Mg, magnesium; NOx, the stable oxidation products of NO, NO2 + NO3 = NOx; NAG, N-acetyl-β-glucosaminidase. P < 0.05 compared with the control group (*), compared with the HP group (†), and compared to the L + HP group (‡).
There was no significant change in nNOS expression in all of the experimental groups when compared with the controls.

Because iNOS(+) fluorescence signal could be found in the cortex and outer medulla of normal control rats (see Fig. 6B), iNOS stained strongly in the medulla as a continuous glandular pattern in HP and L + HP + LN rats, whereas the signal in the cortex was the same as those in the controls. In L + HP rats, the iNOS(+) signal was stronger than the controls and formed not only a glandular pattern but also a circular pattern in both the cortex and medulla.

Figure 3 demonstrated in situ formation of NTS in the kidney by immunohistochemical staining. Because NTS was not only a glandular pattern but also a circular pattern in both the cortex and medulla.

Using the amount of MPO to represent the infiltration of neutrophils and macrophages (1), as shown in Fig. 4E, changes in MPO levels were consistent with those of ED1-positive cell infiltration.

l-Arg induced macrophage/monocyte infiltration. Figure 4 shows micrographs of anti-ED1 antibody staining, demonstrating infiltration of macrophages/monocytes in the kidney. Only a few ED1-positive cells were found in kidney sections of the control [0.8 ± 0.5 cells/high-power field (HPF)] (Fig. 4A), L (1.1 ± 0.6 cells/HPF), LN (0.9 ± 0.5 cells/HPF), and L + LN groups (data not shown). In the L + HP group, the ED1 cells/HPF was increased to 23.7 ± 2.1 and was mainly located in the peritubular interstitium (Fig. 4C). The number of ED1 cells/HPF was significantly reduced to 8.0 ± 1.6 in the L + HP + LN group (data not shown), similar to the 10.3 ± 2.6 cells/HPF seen in the HP group (Fig. 4B) and 9.6 ± 2.5 cells/HPF of the HP + LN group (data not shown). However, in all of the HP-treated groups, some of the ED1-positive cells gathered around the CaOx crystals and formed bigger areas of positive signals, which made cell counting more difficult.

Using the amount of MPO to represent the infiltration of neutrophils and macrophages (1), as shown in Fig. 4E, changes in MPO levels were consistent with those of ED1-positive cell infiltration.

l-Arg caused increased oxidative enzyme proteins and cell injury. Expression of gp91phox, a major component of NAD(P)H oxidase, was increased in the medulla in the HP and L-arginine methyl ester (L-NAME). No obvious CaOx crystal deposition was seen in the control and L-Arg (L), L-NAME (LN), and L + LN groups (data not shown). Reduced from ×40 magnification.

### Table 2. Indexes of oxidative stress in the eight study groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L</th>
<th>LN</th>
<th>L + LN</th>
<th>HP</th>
<th>HP + LN</th>
<th>L + H</th>
<th>L + HP</th>
<th>L + HP + LN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal CL, counts/10 s</td>
<td>2.912±343</td>
<td>3.529±459</td>
<td>2.856±298</td>
<td>2.815±312</td>
<td>5.348±385*</td>
<td>4.981±284*</td>
<td>6.492±424*†</td>
<td>5.487±323*‡</td>
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<tr>
<td>Urinary MDA, μmol/g Cr</td>
<td>12.8±1.1</td>
<td>16.8±1.4</td>
<td>13.3±1.2</td>
<td>13.5±1.2</td>
<td>72.0±10.8*</td>
<td>66.4±8.3*</td>
<td>94.5±7.4*†</td>
<td>69.5±9.4*‡</td>
<td></td>
</tr>
<tr>
<td>Cortex GSH, nmol/mg protein</td>
<td>71.7±7.6</td>
<td>61.6±5.6</td>
<td>68.8±5.9</td>
<td>65.2±6.8</td>
<td>60.5±2.4*</td>
<td>60.2±2.4*</td>
<td>54.0±2.5*†</td>
<td>59.0±2.3*‡</td>
<td></td>
</tr>
<tr>
<td>GSSG, nmol/mg protein</td>
<td>25.7±1.6</td>
<td>27.7±1.5</td>
<td>25.8±1.5</td>
<td>26.4±1.5</td>
<td>26.4±1.0</td>
<td>27.7±1.5</td>
<td>28.2±2.3</td>
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<tr>
<td>Redox ratio, %</td>
<td>42.2±2.8</td>
<td>47.7±1.8</td>
<td>42.9±1.7</td>
<td>44.7±2.4</td>
<td>46.7±1.0*</td>
<td>47.9±1.5*</td>
<td>51.1±1.7*†</td>
<td>48.4±1.8*‡</td>
<td></td>
</tr>
<tr>
<td>Medulla GSH, nmol/mg protein</td>
<td>60.3±5.4</td>
<td>51.0±4.5</td>
<td>57.1±5.2</td>
<td>55.7±4.8</td>
<td>47.5±2.4*</td>
<td>45.3±2.6</td>
<td>33.1±4.0*</td>
<td>48.9±5.0*‡</td>
<td></td>
</tr>
<tr>
<td>GSSG, nmol/mg protein</td>
<td>24.1±2.1</td>
<td>25.9±2.1</td>
<td>24.5±2.0</td>
<td>24.7±1.9</td>
<td>34.5±2.4*</td>
<td>31.7±2.6</td>
<td>39.4±3.1*</td>
<td>30.9±2.2*†</td>
<td></td>
</tr>
<tr>
<td>Redox ratio, %</td>
<td>44.5±3.1</td>
<td>50.5±3.2</td>
<td>46.2±2.8</td>
<td>47.0±2.4</td>
<td>58.9±1.9*</td>
<td>58.3±1.8*</td>
<td>70.4±3.3*</td>
<td>55.8±2.4*‡</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 8 rats in each group. Shown is free radical formation on the left kidney surface, urinary lipid peroxides, and glutathione redox balance in the different groups. GSH, reduced glutathione; GSSG, oxidized glutathione; CL, chemiluminescence; MDA, malondialdehyde. Redox ratio of glutathione was calculated by [2GSSG/(GSH + 2GSSG)]. P < 0.05 compared with the control group (*), compared with the HP group (†), and compared with the L + HP group (‡).
of full-length and cleaved PARP in the L + HP and HP groups were increased significantly compared with those of the control group, and those in the L + HP group were higher ($P < 0.05$).

gp91phox stained strongly in the medulla as a continuous glandular pattern in HP-treated rats, whereas the signal in the cortex was the same as in the controls (Fig. 6A). In L + HP rats, the gp91phox(+) signal was stronger than in the controls and formed not only a glandular pattern but also in the interstitium of both the renal cortex and medulla (Fig. 6A).

**DISCUSSION**

As observed in hyperoxaluric rats, HP-induced hyperoxaluria and renal CaOx crystal accumulation was associated with increased LPO in the hyperoxaluric kidney and renal tubular injury. LPO may result from increased superoxide formation and decreased antioxidant system in hyperoxaluric kidneys (13, 19). CaOx crystal deposition was associated with macrophage infiltrat-
tion, and MPO levels increased (19), which might have contributed to the nitrosative stress in hyperoxaluric kidney. Through coin treatment with L-Arg and HP, more NTS-, ED1-, and iNOS-positive cells gathered around the increased CaOx crystals in the kidney. Increased oxidative stress was indicated by reduced antioxidant compound (GSH), increased O$_2^-$ production, and oxidative injury (MDA and PARP), which can be partially reversed by the co-administration of L-NAME. Therefore, these results clearly showed that chronic L-Arg treatment is not beneficial to hyperoxa-

![Fig. 3. Changes in renal nitrotyrosine (NTS) expression in the different groups. The photographs are representative results from an untreated control rat (A), a rat treated with HP (B), and a rat treated with L + HP (C) by immunohistochemical staining. All sections were evaluated by light microscopy. NTS-positive areas were stained brown. The black arrows indicate crystal deposition in the tubular lumen. Reduced from ×200. *P < 0.05 compared with the control group.]

![Fig. 4. L-Arg induced cell infiltration. Representative micrographs show the presence of the monocyte/macrophage antigen ED1 in renal sections from each group. ED1-positive cells are indicated by arrows. A: controls. B: rat treated with HP. C: rat treated with L-arginine and HP. D: statistical results for each group. E: levels of myeloperoxidase (MPO) in the renal cortex and medulla in each group. Reduced from ×200. P < 0.05 compared with the control group (*), compared with the HP group (#), and compared with the L + HP group (†).]
aluric kidneys with CaOx crystal deposition but instead enhances intrarenal oxidative and nitrosative stress. However, L-Arg administration did not change urinary ion activity and excretion of urine CaOx sediments. As such, grade II CaOx crystals were still found in the kidneys of L + HP + LN rats. L-NAME administration also did not change the urinary ion activity of urine CaOx sediments, since grades I-II crystals were found in the kidneys of HP + LN rats.

Aside from phagocytic cells, the expression of NAD(P)H oxidase has also been detected in kidney cells such as proximal tubular cells, glomerular mesangial cells and cells of the thick ascending loops of Henle (36). NAD(P)H oxidase is a multi-component enzyme complex, and both gp91phox and Rac1 are critical components of endothelial NAD(P) oxidase (37). It is the primary source of \( \text{O}_2^- \) in the kidneys under physiological conditions, and the induction of oxidative stress or overproduction of \( \text{O}_2^- \) often depends on its increased expression or activity (21).

Involvement of NAD(P)H oxidase in the production of free radicals when renal epithelial cells are exposed to oxalate ions or crystals has been reported in a cell culture study (36). In this study, increased NAD(P)H oxidase protein expression and \( \text{O}_2^- \) production were found in the kidneys of L + HP rats, which might have led to more oxidative injury than those treated with HP only but could still be reversed by L-NAME cotreatment. However, gp91phox protein expression showed no significant change in the kidneys of L-Arg-treated rats. We hypothesize that, to enhance NAD(P)H oxidase expression after L-Arg administration, an oxidative stress needs to be present, such as reduced GSH content. But the exact mechanisms need further evaluation.

eNOS has been found to be an important source of vascular \( \text{O}_2^- \) excess in adjuvant-induced arthritis (10). In certain conditions, it may cause uncoupling, which generates \( \text{O}_2^- \), and uncoupled eNOS is reported to be a possible prominent source of endothelial \( \text{O}_2^- \) in hypertension or diabetes (27, 34). It has been reported that eNOS generates \( \text{O}_2^- \) rather than NO when exposed to oxidant stress, including ONOO⁻, or when deprived of its cofactor BH₄ or substrate L-Arg (10, 22).
In the present study, t-Arg administration did not cause an increase in eNOS expression or $O_2^-$ production in the kidneys of normal rats. However, L + HP rats have more eNOS protein expression in the kidneys and reduced $O_2^-$ production when incubated with BH$_4$. This suggests that BH$_4$ may be deficient in the kidneys of L + HP rats and that its addition reverses t-Arg-induced $O_2^-$ increase. Combining these results, we speculate that increased $O_2^-$ production by NAD(P)H oxidase may decrease BH$_4$ bioavailability (23) and results in eNOS uncoupling, leading to further amplification of oxidative stress in the kidneys of L + HP rats.

In addition to NAD(P)H oxidase and uncoupled eNOS, other mechanisms may contribute to oxidative stress in hyperoxaluric kidneys, such as xanthine oxidase (30) and mitochondria (25). We examined the expression of xanthine oxidase in our preliminary study and found no significant change (data not shown). Meimaridou et al. (25) had reported that mitochondrial-derived superoxide was the source of oxidative stress in renal epithelial cells treated with CaOx crystals.

The additive role of oxidative and nitrosative stress in epithelial cell injury has been examined by Peresleni et al. (28). Exposure of epithelial BSC-1 cells to oxidant stress (0.5 mM H$_2$O$_2$) resulted in increased NO release and coincided with the appearance of immunodetectable iNOS expression. This may be the mechanism of increased iNOS protein expression in the cortex of L + HP rats. Combining the findings of immunolabeling for NTS, ED1, and iNOS, we speculate that the enhanced iNOS expression may derive from two different origins: one from the renal tubules, such as the proximal tubules and inner medullary collecting duct (9), and the other is from macrophages/monocytes, which forms a circular shape and goes to the interstitium.

NO generated by iNOS, especially its metabolite peroxynitrite (as detected with NTS antibodies), is cytotoxic to renal tubular epithelium (9). Enhanced NTS formation in the kidneys and urine without an increase in urinary NOx and cGMP excretion was observed in L + HP rats when compared with the t-Arg-treated group, and that was the possible mechanism that NO reacted with superoxide yielding peroxynitrite, which itself interacted with tyrosine residues to form NTS and then to be sequestered in tissues or excreted in the urine rather than metabolized (17).

Enhanced iNOS protein expression in the renal cortex of L + HP rats can be reversed when t-NAME is used as a cotreatment. We speculate that t-Arg administration to induce iNOS protein expression and nitrosative stress in the kidneys needs oxidative stress to exist in those kidneys already. In stress-induced gastric damage, nuclear factor-$k$B (NF-$k$B) is activated by reactive oxygen species (ROS), which then induces iNOS gene expression (18). Whether the increased iNOS protein expression after chronic t-Arg administration is through NF-$k$B activation needs further evaluation.

During induction of CaOx nephrolithiasis, infiltration of ED1-positive cells into the kidneys and these cells surrounding CaOx crystals have been found in animal models (13, 19). t-Arg administration reduces renal macrophage infiltration in obstructive nephropathy after a 3-day t-Arg administration (32). However, in the present study, renal ED1-positive cell infiltration was not abolished in L + HP rats after a 6-wk t-Arg administration. Instead, ED1 infiltration was partially reversed after cotreatment with t-NAME. Therefore, combined with earlier data, we hypothesize that the critical factor that determines the effect of t-Arg administration is the presence of oxidative stress.

For example, Pragasam et al. (30) reported restored antioxidant enzyme levels to normal and no stone formation in the kidneys after t-Arg cosupplementation to 0.75% ethylene glycol-treated rats for 28 days. In addition to oxalate, CaOx crystals are associated with multinucleated giant cells and macrophages that are injurious to renal epithelial cells (19). Therefore, if renal oxidative stress is not increased and no CaOx crystal accumulates in hyperoxaluric kidney, t-Arg cotreatment has an antilithic effect. We performed the same experiments on day 14 in a preliminary study (data not shown), and the results were consistent with those of Pragasam et al. (30).

Our findings are consistent with those found in the atherosclerotic model in which severity of atherosclerosis determines the t-Arg treatment to be beneficial or harmful to atherosclerosis. t-Arg (2.25%) treatment of severely atherosclerotic rabbits (induction for 24 wk) leads to increased $O_2^-$ production (34), whereas reduced $O_2^-$ and decreased plaque progression were found in rabbits after 12 wk induction of atherosclerosis and cotreated with t-Arg (2.25%) in Böger’s study (3). The source of $O_2^-$ in severely atherosclerotic aorta may be NAD(P)H oxidase, xanthine oxidase, or uncoupled eNOS (3, 34).

In summary, the present study indicates that t-Arg administration can enhance NAD(P)H oxidase, eNOS, and iNOS protein expression in the hyperoxaluric kidneys with massive CaOx crystal deposition. Increased ROS production by NAD(P)H oxidase may result in eNOS uncoupling and leads to further amplification of oxidative stress in the kidneys. Increased macrophage/monocyte infiltration and iNOS expression in the kidneys are likewise associated with increased nitrosative stress in t-Arg-treated hyperoxaluric rats. This increased renal oxidative and nitrosative stress can further reduce renal antioxidant ability and result in more tubular damage and CaOx crystal accumulation in the kidneys. Increased CaOx crystal accumulation in the kidney after long-term induction of nephrolithiasis may cause excessive ROS production, which may result in the cotreatment of t-Arg lacking an antilithic effect.

GRANTS

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