Enalapril Prevents Tubulointerstitial Lesions by Hyperoxaluria

Jorge Eduardo Toblli, Inés Stella, Elena de Cavanagh, Margarita Angerosa, Felipe Inserra, León Ferder

Abstract—Hyperoxaluria is a recognized cause of tubulointerstitial lesions, and this could contribute to development of hypertension and chronic renal failure. Enalapril has been effective against the progression of tubulointerstitial lesions in various animal models. The aim of the present study was to evaluate the usefulness of enalapril on the tubulointerstitial damage produced by oxalates. Two-month-old male Sprague-Dawley rats were separated into 4 groups, control with tap water (G1), hyperoxaluric (G2), hyperoxaluric+enalapril (G3), enalapril (G4), for 4 weeks. G2 and G3 rats were given 1% ethyleneglycol (ETG, precursor for oxalates), and G3 and G4 rats were given enalapril 20 mg/L in drinking water. At the end of the study, we evaluated renal tubulointerstitial lesions by a semiquantitative score. Urine albumin excretion, serum and urine nitric oxide production, tubulointerstitial immunostaining by α-smooth muscle actin, transforming growth factor-β1, and collagen type III were measured. Rats belonging to the hyperoxaluric group treated with enalapril (G3) showed fewer tubulointerstitial lesions (1.3±0.2 versus 3±0.2; P<0.01), lower urine albumin excretion (8±2 mg/d versus 25±2 mg/d; P<0.01), less percentage of α-smooth muscle actin in renal interstitium (2±0.4% versus 13.5±2.4%; P<0.01), less percentage of transforming growth factor-β1 in tubulointerstitial area (3.3±1% versus 13.3±2.1%; P<0.01), less percentage of collagen type III interstitial deposition (0.7±0.5% versus 7±2.6%; P<0.01), and increased NO production in serum as well as urine (both P<0.01), when compared with the hyperoxaluric group not treated with enalapril (G2). Considering these data, we believe that enalapril, by several mechanisms of action, could provide an important benefit in the prevention of inflammatory response, transforming growth factor-β1 tubulointerstitial production, collagen type III interstitial deposition, and finally, the progressive tubulointerstitial fibrosis caused by oxalates. (Hypertension. 1999;33[part II]:225-231.)

Key Words: enalapril ■ hyperoxaluria ■ tubulointerstitial lesions ■ renal fibrosis ■ renin-angiotensin system.

Recent studies have revealed the importance of the renal interstitium in the progression of renal failure. One of the most important factors is tubulointerstitial (TI) fibrosis.1 Fibrosis is caused by a combination of both increased extracellular matrix (ECM) protein synthesis and inhibition of ECM degradation. Interstitial fibrosis is characterized by accumulation of ECM proteins generally found in the interstitium (such as collagen I, collagen III, and fibronectin) and the de novo appearance of ECM proteins such as collagen IV and laminin. Overexpression of protease inhibitors, including the tissue inhibitors of metalloproteinases (TIMP) or plasminogen activator inhibitors (PAI), is the most consistent change in the ECM degradation pathway. Moreover, expression of the fibrogenic cytokine, transforming growth factor-β1 (TGF-β1), is upregulated. In addition, the interstitium is infiltrated by cells of the monocyte/macrophage lineage. These cells appear to be an important source of TGF-β1. The fundamental mechanisms involved in the recruitment of circulating monocytes into the interstitium remain largely unknown.2

Numerous studies have reported the relationship between TGF-β1 and ECM production.3,4 It is currently recognized that TGF-β1 (1) induces α-smooth muscle actin expression in mesangial cells; (2) modulates fibroblast-myofibroblast transformation; (3) stimulates protein synthesis of types I, III, and V collagen, fibronectin, and proteoglycans; (4) is upregulated by TIMP; and (5) participates in apoptotic process.5 In the kidney several cells express TGF-β1, such as mesangial cells, epithelial tubular cells, macrophages, fibroblasts, and myofibroblasts.

The renin-angiotensin system (RAS) has an important role in the development of TI fibrosis in experimental models, because ACE inhibitors as well as angiotensin II (Ang II) type 1 (AT1) receptor antagonists can reverse it.6–8 Hyperoxaluria (Hox) is a well-known cause of renal stone disease and TI damage. In hyperoxaluric states, oxalate accumulates in renal tubular cells and leads to the

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development of continuous TI inflammation. The purpose of this study was to evaluate the effects of an ACE inhibitor, enalapril (E), on the TI damage produced by Hox.

**Methods**

All experiments were approved by the Hospital Alemán Ethic Committee and the Teaching and Research Committee, and followed the NIH Guide for the Care and Use of Laboratory Animals. Two-month-old male Sprague-Dawley rats (Facultad de Cs. Veterinarias, Universidad Nacional La Plata; Charles River Laboratories, Houston, Tex), initially weighing 250 to 300 g, were housed in metabolic cages at a room temperature of 21 ± 2°C and in a 12 hour light/darkness cycle (7 AM to 7 PM). After 7 days they were divided into 4 groups: control group (G1, n = 6), ETG group (G2, n = 6), ETG+E group (G3, n = 6), and E group (G4, n = 6). All animals were allowed to drink regular tap water and were fed standard rat chow (16% to 18% protein, Cargill-Argentina) ad libitum. For 4 weeks, ETG 1% (as a precursor for oxalates) was administered to G2 and G3, and E 20 mg/L was administered to G3 and G4, both in drinking water. Urine was collected for 24 hours under 2 mL toluene (Aldrich Chemical Co.) for pH, creatinine, oxalate, and urine albumin (UAE) determinations. After 4 weeks all animals were euthanatized (pentobarbital, 40 mg/kg body weight IP), and the kidneys were harvested for histologic studies. Rats were bled through the aorta before euthanasia, and blood samples were used for creatinine and NO₂⁻NO₃ determinations.

**Blood Pressure Measurement**

Systolic blood pressure (SBP) was measured by tail-cuff plethysmography while rats were quietly restrained in a plastic chamber. A minimum of 3 measurements was taken at each session, and the SBP registered was the average of the 3 readings of 3 minutes each.

**Biochemical Procedures**

Serum and urine creatinine, urine pH, oxalate and urine albumin concentrations were measured by standard techniques. Nitric oxide (NO) production was evaluated by measuring plasma and urine nitrite plus nitrate with a colorimetric assay based on the Greiss reaction.

**Kidney Processing and Examination**

Kidneys were perfused with saline solution through the abdominal aorta until free of blood. Decapsulated kidneys were cut longitudinally and fixed in phosphate-buffered 10% formaldehyde (pH 7.2) and embedded in paraffin. Three-micron sections were cut and stained with hematoxylin-eosin (H&E), periodic acid-Schiff reagent (PAS), and Mason’s trichrome.

**Immunolabeling and Optical Microscopy**

Immunolabeling of specimens was carried out by a modified avidin-biotin-peroxidase complex technique Vectastain ABC kit (Universal Elite, Vector Laboratories) and the specimens were handled as described previously. Renal α-smooth muscle actin (α-SMA), collagen (COL) III, and TGF-β1 were quantified with use of anti-mouse α-SMA, clone no. 1A4, lot no. 107F-4806 (Sigma

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**TABLE 1. Parameters on Week 4**

<table>
<thead>
<tr>
<th></th>
<th>G1 (n=6)</th>
<th>G2 (n=6)</th>
<th>G3 (n=6)</th>
<th>G4 (n=6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mm Hg)</td>
<td>123.3±.5</td>
<td>124.±6</td>
<td>124.3±.6</td>
<td>123.5±.6</td>
<td>.596</td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>1.2±.07</td>
<td>1.1±.08</td>
<td>1.1±.07</td>
<td>1.2±.08</td>
<td>.629</td>
</tr>
<tr>
<td>Serum NO₂⁻NO₃ (µmol/L)</td>
<td>9.2±.15</td>
<td>9.1±.11</td>
<td>13±.7</td>
<td>10.7±.3</td>
<td>.002*</td>
</tr>
<tr>
<td>Urine pH</td>
<td>6.7±.16</td>
<td>6.3±.12</td>
<td>6.6±.2</td>
<td>6.7±.16</td>
<td>.301</td>
</tr>
<tr>
<td>Urine oxalate (µg/g BW/d)</td>
<td>1.8±.12</td>
<td>12.1±.73</td>
<td>13.2±.9</td>
<td>13.2±.9</td>
<td>&lt;.01†‡</td>
</tr>
<tr>
<td>UAE (mg/d)</td>
<td>0</td>
<td>25±2</td>
<td>8±.2</td>
<td>0</td>
<td>&lt;.01*</td>
</tr>
<tr>
<td>Urine NO₂⁻NO₃ (µmol/L)</td>
<td>1.8±.09</td>
<td>1.7±.11</td>
<td>2.4±.17</td>
<td>2.6±.17</td>
<td>&lt;.01*</td>
</tr>
</tbody>
</table>

BW indicates body weight.

*P<.01=G2 vs G3; †P<.01=G2 vs G1, G4; ‡P<.01=G3 vs G1, G4.
Chemical Co.) monoclonal antibodies; anti-COL III, lot AM1670696 (Biogen), and anti-human TGF-β, lot 496143 dilution 1:400 (Chemicon) monoclonal antibodies, with protease 0.1% for 8 minutes and then washing with distilled water.

**Morphologic Analysis**

Twenty histologic sections were studied in each animal by an image analyzer (Bioscan-OPTIMAS). Morphologic analyses were performed with the observer blind to the animal treatment group. TI lesions were

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**Figure 2.** G3 (ETG+E) rat. Renal tissue preserved from crystal injury without tubular damage or interstitial cell infiltrates (H&E, ×400).

**Figure 3.** G2 (ETG) rat. Section of renal tissue showing large area with strong positive staining (in brown) in peritubular cells (α-SMA, ×200).

**Figure 4.** G3 (ETG+E) rat. Renal tissue with positive staining (in brown), just in vessel walls (α-SMA, ×100).
graded: 0 = absent; 1 = mild; 2 = moderate; 3 = severe; or 4 = very severe. Sections were evaluated for percentage of: (1) α-SMA in renal TI; (2) COL III in renal TI; (3) TGF-β1 in renal TI.

**Statistical Method**

Values were expressed as mean ± SEM. All statistical analysis were performed with absolute values and processed through GraphPad Prism, version 2.0 (GraphPad Software, Inc.). All comparison among groups was performed by ANOVA. Difference of mean values between two groups was assessed by the two-tail t test. A value of P < 0.05 was considered significant.

**Results**

Urine pH, urine oxalate, urine NO2⁻ and NO3⁻, and blood pressure did not differ among groups, and albuminuria was

**Figure 5.** G2 (ETG) rat. Renal tissue with a big area with strong positive staining (in brown) within the tubular and peritubular cells. Note oxalate calcium crystals in tubular lumens (TGF-β1, ×200).

**Figure 6.** G3 (ETG+E). Renal tissue with a small area with positive staining (in brown) within the tubular and peritubular cells (TGF-β1, ×200).

**Figure 7.** G2 (ETG) rat. Section of renal tissue with a big area with strong positive staining (in brown) between tubular cells (COL III, ×200).
not detected at baseline. At the end of the 4th week, a marked elevation of urine oxalate occurred in rats from G2 (ETG) and G3 (ETG+E) (Table 1). SBP, urine pH, and creatinine clearance did not change significantly in any group at the end of the experiment (Table 1). UAE rose in G2 (ETG) compared with the other groups (P<0.01), and was higher than in G3 (ETG+E) (Table 1). Rats in G3 (ETG+E) showed an increase in serum and urine NO$_2$+NO$_3$ production (as an index for NO production) compared with G2 (ETG) (Table 1). Light microscopy showed that animals from G2 had diffuse TI lesions with large amounts of oxalate crystals in the tubular lumen and in epithelial tubular cells (Figure 1). In the same group, proximal tubule epithelial cells exhibited vacuoles and hydropic changes. Tubule epithelial cell atrophy and mononuclear cell infiltrate were seen in the interstitium (Figure 1). In the same group, peritubular and tubular cells and in interstitial COL III was detected by immunostaining in rats in G2 (ETG) (Figures 3, 5, and 7 and Table 2). Rats from G3 (ETG+E), presented small (P<0.01) amounts of α-SMA, TGF-β1, and COL III as shown in Figures 4, 6, and 8 and Table 2.

**Discussion**

Hyperoxaluric animals not treated by E (G2) had serious damage in epithelial cells and in the renal interstitium. As previously reported by us$^{12}$ and others,$^{13}$ hyperoxaluric animals that received E (G3) had significantly fewer TI changes.

The mechanism by which hyperoxaluria and increased intracellular oxalate produce tubular cell damage is unknown. Oxalate is transported bidirectionally in the tubular cell, and its accumulation inside the cell produces changes in the activity of several enzymes (eg, lactate dehydrogenase, malate dehydrogenase, pyruvate kinase).$^{14,15}$ Studies with LLC-PK$_1$ cells (a line of renal epithelial cells with characteristics of proximal tubular cells) revealed that oxalate in a high concentration acts as a cellular toxin by increasing free-radical production,$^{16}$ a process that could be directly injurious or a catalyst for the start of various inflammation pathways (Figure 9).

Interstitial inflammation, edema, or fibrosis can impair glomerular arteriolar outflow, lead to increased intraglomerular hypertension, and induce proteinuria.$^{17}$

Significant cortical renal interstitium increases in COL III deposition, the earliest collagen type to appear in fibrotic processes, were seen in G2 (no E treatment). This element of renal fibrosis and proteinuria were blunted by E administration (Figure 8; Tables 1 and 2). These findings suggest that the RAS is involved in the pathophysiology of hyperoxaluric TI lesions.

Studies in humans and animals indicate that Ang II can induce TGF-β1 and alter ECM production by increasing its deposition.$^{18–21}$ Also, TGF-β1 inhibits matrix degradation, upregulates the integrin matrix-adhesion molecules, and leads to chemotraction of fibroblasts and monocytes.$^{22}$ It regulates type I, type III, and type VI collagen, fibronectin, and laminin$^{23,24}$; it also influences the transcription of α-SMA, which participates in the transformation of fibroblast to myofibroblast.$^{25}$

In a model of urinary tract obstruction, E significantly reduced the interstitial volume and the amount of renal cortical collagen.$^7$ Klahr et al$^3$ and Pimentel et al$^{19}$ proposed that urinary tract obstruction activates the RAS and stimulates TGF-β1 synthesis by tubular epithelial cells. Hyperoxaluric animals in our study have marked deposition of TGF-β1 in the interstitium (Figure 5 and Table 2).

### Table 2. Morphological Analysis of Renal TI Tissue on Week 4

<table>
<thead>
<tr>
<th></th>
<th>G1 (n=6)</th>
<th>G2 (n=6)</th>
<th>G3 (n=6)</th>
<th>G4 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SEM</td>
<td>Control</td>
<td>ETG</td>
<td>ETG+E</td>
<td>E</td>
</tr>
<tr>
<td>TI lesions score</td>
<td>0.2±0.1</td>
<td>3±0.2*</td>
<td>1.3±0.2</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>Ti α-SMA (%)</td>
<td>0.5±0.3</td>
<td>13.5±2.4*</td>
<td>2±0.4</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>Ti TGF-β1 (%)</td>
<td>3.8±1.1</td>
<td>13.3±2.1*</td>
<td>3.3±1</td>
<td>5.3±2.1</td>
</tr>
<tr>
<td>Ti COL III (%)</td>
<td>0.3±0.2</td>
<td>7±2.6*</td>
<td>0.7±0.5</td>
<td>0.3±0.4</td>
</tr>
</tbody>
</table>

*$p<.01=G2$ vs G1, G3, G4.
E and captopril increase antioxidant enzyme levels in various organs of the mouse, including the kidney. This could be another mechanism by which ACE inhibitor protects against oxalate cytotoxicity and against tubular and interstitial injury.

We conclude that E protects the kidney from the TI damage produced by oxalate by reducing Ang II production, TGF-β1 synthesis and deposition, COL III interstitial deposition, and thus progressive TI fibrosis.

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References


Figure 9. Pathways by which calcium oxalate produces tubulointerstitial lesions. Calcium oxalate crystals produce cell injury in proximal tubular epithelium followed by monocyte/macrophage infiltration. Local inflammation contributes to release Ang II, different cytokines, including TGF-β1, as well as Ang II stimulates the transformation from fibroblasts in myofibroblasts, both types of cells produce extracellular matrix protein such as COL III starting the fibrogenetic process.

These changes, in addition to macrophage parenchyma infiltration, can cause renal tissue progressive fibrosis (Figure 9).

Hyperoxaluric rats treated with E exhibit a very modest amount of TGF-β1 in epithelial tubular cells as well as in the interstitium (Figure 6 and Table 2). In the present studies, obstruction to urine flow because of intratubular oxalate precipitation may have been one of the mechanisms leading to interstitial fibrosis and mononuclear cell infiltration. Mononuclear cells can significantly increase local TGF-β1 synthesis. Because E reduced the intensity of staining for TGF-β1, our data suggest that RAS is involved in hyperoxaluric TI fibrosis.

Similar results with ACE inhibition were observed in rats with chronic immune complex nephritis, in which quinapril decreased renal cortical mRNA levels for TGF-β1, fibronectin, and I, II, and IV collagen. ACE inhibition is associated with a significant decrease in monocyte/macrophage infiltration of the kidney with ureteral obstruction. Inhibition of ACE may also exert a beneficial effect through its action to increase local amounts of bradykinin. Bradykinin is a potent stimulus for the release of NO, which ameliorates the TI lesions caused by ureteral obstruction by reducing monocyte/macrophage infiltration. Administration of L-arginine, a precursor of NO, decreases obstruction to urine flow because of intratubular oxalate precipitation. Bradykinin is a potent stimulus for the release of NO, which ameliorates the TI lesions caused by ureteral obstruction by reducing monocyte/macrophage infiltration. Administration of L-arginine, a precursor of NO, decreases obstruction to urine flow because of intratubular oxalate precipitation.


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