Effects of Amlodipine on Tubulointerstitial Lesions in Normotensive Hyperoxaluric Rats

Jorge Eduardo Toblli, León Ferder, Margarita Angerosa, Felipe Inserra

Abstract—Although controversial, a number of reports have suggested that calcium antagonists can retard or prevent the progression of various renal diseases in experimental models. Nevertheless, there are few data related to tubulointerstitial changes in these studies. On the other hand, hyperoxaluria is a recognized cause of tubulointerstitial lesions, and this could contribute to the development of hypertension and chronic renal failure. The aim of the present study was to evaluate a possible beneficial effect of amlodipine, a 1,4-dihydropyridine class of calcium antagonist, in a model of primary tubulointerstitial lesion produced by hyperoxaluria. Two-month-old male Sprague-Dawley rats were separated into 4 groups for a 4-week period: G1 (control; tap water only); G2 (hyperoxaluric); G3 (hyperoxaluric plus amlodipine treatment); and G4 (amlodipine treatment). G2 and G3 rats were given 1% ethylene glycol (a precursor for oxalates) in drinking water, and G3 and G4 rats were given amlodipine 2 mg · kg⁻¹ · d⁻¹ by gavage. At the end of the study, we evaluated by semiquantitative scores (0 to 4) the different renal tubulointerstitial lesions, urinary albumin excretion, renal function by creatinine clearance, and blood pressure. Rats belonging to the hyperoxaluric group treated with amlodipine (G3) had fewer tubulointerstitial lesions, as follows: (1) inflammatory infiltrate score: 3.31 ± 0.07 versus 0.23 ± 0.12; P < 0.05; (2) tubular atrophy score: 3.33 ± 0.33 versus 0.50 ± 0.22, P < 0.05; (3) interstitial fibrosis score: 2.76 ± 0.34 versus 0.31 ± 0.16, P < 0.05; (4) oxalate deposits score: 3.66 ± 0.33 versus 0.09 ± 0.08, P < 0.05; (5) lower urinary albumin excretion (11.3 ± 2 versus 27 ± 4.5 mg/d, P < 0.01); and (6) higher creatinine clearance (1.22 ± 0.08 versus 1.13 ± 0.08, P < 0.01) compared with the hyperoxaluric group untreated with amlodipine (G2). On the other hand, there were no significant changes in blood pressure in any group. In view of these data, we suggest that amlodipine, probably by nonhemodynamic mechanisms of action, can provide an important benefit in the prevention of epithelial tubular cell injury and inflammatory response and therefore in the prevention of the progressive tubulointerstitial fibrosis caused by oxalates. (Hypertension. 1999;34(part 2):854-858.)

Key Words: calcium antagonists ■ amlodipine ■ hyperoxaluria ■ tubulointerstitial lesions ■ fibrosis

Although there is a large body of studies on the subject, the available data on the ability of calcium antagonists (CAs) to retard or prevent the progression of renal diseases in animal models are controversial. A number of reports have suggested that CAs are protective in experimental renal insufficiency1,2; nevertheless, other investigators have failed to demonstrate beneficial effects of CA in diverse experimental models of chronic renal failure.3,4

Conflicting results exist concerning the protective effect in experimental glomerulopathies,5,6 as well as in different hypertension models.7,8 Kloke et al9 recently published an important review about the controversial risk/benefit relationship in animal models and the clinical use of CAs in renal disease. In spite of this, there are few data related to tubulointerstitial (TI) changes in these studies, and nothing has been published yet in a specific TI lesion model.

Chronic hyperoxaluric states are a recognized cause of TI disease. In the ethylene glycol (ETG)–induced hyperoxaluria animal model, we and other authors have found serious damage to the renal TI. The lesions were characterized by tubular epithelial cell necrosis, calcium oxalate crystal deposits in tubular lumens, inflammatory infiltrates, and proliferation of resident interstitial cells, such as fibroblasts and their transdifferentiation to myofibroblasts, and therefore by an increase in extracellular matrix and fibrosis.10,11 Significant protection against these lesions was conferred by an ACE inhibitor, enalapril.11

The present study was conducted to evaluate a possible beneficial effect of amlodipine, a 1,4-dihydropyridine class of CA, in this model of primary TI lesions produced by hyperoxaluria.

Methods

All experiments were approved by the Hospital Alemán Ethics 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 (Charles River Laboratories, Wilmington, Mass) initially weighing 250 to 300 g were housed in metabolic cages at a room temperature of 21±2°C with a 12-hour light/dark cycle (7 AM to 7 PM). After a 7-day period, they were divided into 4 groups: control group (G1, n=6), ETG group (G2, n=6), ETG plus amlodipine (ETG+A) group (G3, n=6), and amlodipine-only 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. During a 4-week period, ETG was administered to G2 and G3 rats, and amlodipine 2 mg·kg⁻¹·d⁻¹ was administered to G3 and G4 rats by gavage. Twenty-four-hour urine was collected under 2 mL of toluene (Aldrich Chemical Co) for pH, creatinine, oxalate, and urinary albumin concentration was measured by microcentrifuge filters (ultrafree-MC NMWL 5000; Sigma Chemical Co), and urinary albumin concentration was determined with a pH meter (model PHM84, research pH meter; Radiometer Copenhagen). Oxalate was determined by the enzymatic method (Sigma Diagnostics). Creatinine clearance was calculated by a standard formula. Previous urinary concentrations were determined with a pH meter; perinephric fat, kidneys were cut longitudinally and fixed in phosphate-buffered 10% formaldehyde (pH 7.2). The kidneys were embedded in paraffin. Three-micrometer sections were cut and stained with hematoxylin-eosin, periodic acid–Schiff reagent, and Masson’s trichrome.

Morphological Analysis
Twenty separate histological sections, 10 from the cortex and 10 from the medulla, were studied in each animal by use of an image analyzer (Bioscan-OPTIMAS). Morphological analysis was assessed on 10 microscopic fields per section examined at a magnification of ×200, with the observer blind to the animal treatment group, and the data were averaged. TI lesion scores for inflammatory cell infiltrate, tubular atrophy, interstitial fibrosis, and oxalate deposits were each graded according to the following scale: 0=absent; 1=mild (involving ≤25% of each microscopic field); 2=moderate (>25% and ≤50%); 3=severe (>51% and ≤75%); and 4=very severe (>76%).

Statistical Analysis
Values are expressed as mean±SE. All statistical analyses used absolute values and were processed through GraphPad Prism version 2.0 (GraphPad Software, Inc). For parameters with gaussian distributions, all comparisons among groups were performed with ANOVA. The difference of mean values between 2 groups was assessed by a 2-tailed t test. Statistical analysis for histological data was performed by Kruskal-Wallis test (nonparametric ANOVA) and Dunn multiple comparisons test. A value of P<0.05 was considered significant.

Results
There were no significant differences in baseline values of SBP, urine pH, urine oxalate, and UAE between groups (Table 1). However, at the end of the fourth week, a marked elevation of urine oxalate was observed in G2 rats (ETG) and the radial immunodiffusion method (Bind a Rid, Nanorid Products, The Binding Site Ltd).

Kidney Processing and Examination
The abdominal aorta was catheterized, and the kidneys were perfused with saline solution. Perfusion was continued until the renal parenchyma looked extremely pale. After removal of the capsule and perinephric fat, kidneys were cut longitudinally and fixed in phosphate-buffered 10% formaldehyde (pH 7.2). The kidneys were embedded in paraffin. Three-micrometer sections were cut and stained with hematoxylin-eosin, periodic acid–Schiff reagent, and Masson’s trichrome.

Biochemical Procedures
Aliquots of sera and urine were assayed for creatinine by the enzymatic UV method (Randox Laboratories Ltd). Urine pH was determined with a pH meter (model PHM84, research pH meter; Radiometer Copenhagen). Oxalate was determined by the enzymatic method (Sigma Diagnostics). Creatinine clearance was calculated by a standard formula. Previous urinary concentrations were determined by microcentrifuge filters (ultrafree-MC NMWL 5000; Sigma Chemical Co), and urinary albumin concentration was measured by the radial immunodiffusion method (Bind a Rid, Nanorid Products, The Binding Site Ltd).

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TABLE 1. Baseline Data

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<tr>
<td>SBP, mm Hg</td>
<td>121.9±0.4</td>
<td>122.1±0.4</td>
<td>121.7±0.5</td>
<td>121.8±0.5</td>
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<td>Urine pH</td>
<td>6.5±0.14</td>
<td>6.5±0.15</td>
<td>6.5±0.16</td>
<td>6.6±0.12</td>
<td>NS</td>
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<tr>
<td>Urine oxalate, µg·g BW⁻¹·d⁻¹</td>
<td>1.6±0.7</td>
<td>1.7±0.5</td>
<td>1.8±0.8</td>
<td>1.6±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>UAE, mg/d</td>
<td>1.5±0.2</td>
<td>1.4±0.5</td>
<td>1.5±0.6</td>
<td>1.3±0.7</td>
<td>NS</td>
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BW indicates body weight. Values are mean±SE.

TABLE 2. Data at Week 4

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<tbody>
<tr>
<td>SBP, mm Hg</td>
<td>123.2±0.5</td>
<td>124.0±0.7</td>
<td>123.5±0.5</td>
<td>123.0±0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>1.23±0.07</td>
<td>1.13±0.08</td>
<td>1.22±0.08</td>
<td>1.23±0.08</td>
<td>*</td>
</tr>
<tr>
<td>Urine pH</td>
<td>6.6±0.16</td>
<td>6.5±0.12</td>
<td>6.6±0.18</td>
<td>6.7±0.14</td>
<td>NS</td>
</tr>
<tr>
<td>Urine oxalate, µg·g BW⁻¹·d⁻¹</td>
<td>1.8±0.12</td>
<td>12.1±0.73</td>
<td>13.2±0.9</td>
<td>1.9±0.09</td>
<td>†‡</td>
</tr>
</tbody>
</table>

BW indicates body weight.

*P<0.05, G2 vs G1, G3, and G4.
†P<0.05, G2 vs G1 and G4.
‡P<0.05, G3 vs G1 and G4.
G3 rats (ETG + A) (Table 2). No significant changes in SBP or urine pH were observed in any group at the end of the experiment. (Table 2).

G2 rats (ETG) showed an increase in UAE compared with the other groups (P<0.01) and lower creatinine clearance, as shown in Figure 1 and Table 2. In contrast, G3 animals (ETG + A) had a significant reduction in UAE that was statistically different from the levels in G2 rats (ETG) (Figure 1).

Light microscopic examination showed that G2 animals had diffuse TI lesions with large amounts of oxalate crystals in the tubular lumen and epithelial tubular cells (Figure 1). In the same group, proximal tubules in the epithelial cells presented important numbers of vacuoles and hydropic changes. In a large number of tubules, epithelial cell atrophy was observed with significant inflammatory cell infiltrate in the interstitium (Figures 2 and 3). Furthermore, TI lesion scores revealed that G2 rats (ETG) had significantly more damage than rats in the other groups, as shown in Table 3. In contrast, G3 rats (ETG + A) showed considerably fewer lesions in the tubules and interstitium, as described above, especially related to the inflammatory cell infiltrate, which was absent (Figures 4 and 5); therefore, these rats had lower TI lesion scores, as indicated in Table 3.

Discussion
Recently, TI injury in diverse nephropathies has been recognized as one of the most important factors in the progression of renal failure.12,13 Although much progress has been made in our understanding of the process, much more must be learned to prevent progression to end-stage renal disease. Both ACE inhibitors and nonpeptidic receptor antagonists of angiotensin II blunt the pathological changes in some experimental models of TI damage.11,14,15 However, in different animal models, in which CAs demonstrate renal protection, the interstitium is poorly studied.

Progression of TI has been evaluated in only a few reports of glomerular injury or hypertensive lesions, as well as in experimental chronic renal failure, and conflicting results have been shown.1,16,17 Thus far, no data are available with regard to a primary TI disease model.

In the present study using a TI lesion model, we found significant protection was conferred by amlodipine on both epithelial tubular cells and renal interstitial tissue, with scanty inflammatory cell infiltrates and deposit of calcium oxalate crystal in the tubular lumen. Therefore, renal function, as expressed by normal creatinine clearance, was preserved (Table 2).

Recently, we11 demonstrated a dramatic amount of transforming growth factor-β1 (TGF-β1) in the TI area and increased collagen type III in the interstitium in hyperoxaluric rats. TGF-β1, a potent fibrogenetic cytokine, should be involved in the pathogenic pathway to develop tissue lesions by oxalate. However, it is currently unclear which is the primary stimulus to start the pathway for tubular and interstitial lesions by hyperoxaluria. Studies with LLC-PK1 cells (a line of renal epithelial cells with characteristics of proximal tubular cells) revealed that high oxalate concentration acts as a toxin, inducing a marked increase in free-radical produc-

**TABLE 3. TI Lesion Scores**

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<tbody>
<tr>
<td>Inflammatory infiltrate</td>
<td>0.08±0.08</td>
<td>3.31±0.07*</td>
<td>0.23±0.12</td>
<td>0.16±0.10</td>
</tr>
<tr>
<td>Tubular atrophy</td>
<td>0.16±0.16</td>
<td>3.33±0.33*</td>
<td>0.50±0.22</td>
<td>0.14±0.10</td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>0.08±0.08</td>
<td>2.76±0.34*</td>
<td>0.31±0.16</td>
<td>0.07±0.06</td>
</tr>
<tr>
<td>Oxalate deposits</td>
<td>0.03±0.02</td>
<td>3.66±0.33*</td>
<td>0.09±0.08</td>
<td>0.03±0.02</td>
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*P<0.05 vs G1, G3, and G4.
tion, resulting in cellular damage.\textsuperscript{18} High levels of oxalate seem to act to damage the tubular cell by increasing free radical production in a number of ways: they act as a precursor in the generation of a reactive metabolite; they alter mitochondrial function; they modify the activities of various cytosolic enzymes; and they inhibit endogenous free radical–scavenging enzymes.\textsuperscript{18}

Hypoxia produced by injury and loss of peritubular capillaries due to interstitial damage can contribute to overproduction of reactive oxygen species (ROS), especially in high metabolic cellular activity situations like oxalate tubular overload. An additional source of ROS may be inflammatory cell infiltrates, which are present in the lesion focus.

As discussed, there were no significant changes in blood pressure in any group at the end of the experiment. This fact suggests that the mechanism by which amlodipine protects against renal TI lesions is not associated with hemodynamic variations. In support of this hypothesis, nifedipine, a dihydropyridine CA, has demonstrated protection against interstitial lesions in the ischemic injury model by administration of N\textsuperscript{\textdagger}-monomethyl-L-arginine. This effect was unrelated to either renal hemodynamic changes or blood pressure modification.\textsuperscript{19}

A prominent pathway postulated to mediate the renalprotective actions of CA includes their ability to ameliorate injury by retarding renal growth; to attenuate the mitogenic effects of diverse cytokines and growth factors, including platelet-derived growth factor and platelet-activating factor; and to act as a free radical scavenger.\textsuperscript{20,21} Moreover, in cell culture of human smooth muscle cells, it recently has been recognized that felodipine, another dihydropyridine CA, produces inhibition in superoxide production\textsuperscript{22} and prevents glutathione loss.\textsuperscript{21}

Considering that ROS participate in oxalate TI injury\textsuperscript{19} and that this could contribute to initiation of the various inflammation pathways, the role of CA in relation to the interruption of this process could be relevant.

Finally, an additional mechanism to protect epithelial tubular cells could be amelioration of mitochondrial calcium overload, which results in mitochondria dysfunction and eventual cell death.\textsuperscript{23}

The hyperoxaluric animals not treated by amlodipine showed higher UAE. In accordance with various recent studies, proteinuria may participate in the pathogenesis of TI lesions.\textsuperscript{13,24} Moreover, in a very recent study by Eppel \textit{et al}\textsuperscript{25} in which the mechanism by which filtered albumin is retrieved by the postglomerular transcellular pathway across the proximal tubular epithelium is clarified, the authors suggest that this is a potential target for malfunction in renal diseases that increase proteinuria. On the other hand, in our experiment, UAE was significantly lower in the hyperoxaluric rats treated by amlodipine. Although it is well known that amlodipine has no effect on proteinuria, it is recognized that CAs, especially dihydropyridine agents, have effects on the proximal tubule.\textsuperscript{26} Therefore, our findings on UAE, although limited to this particular model of primary TI lesion disease, could illustrate another factor that contributes to a decrease in TI injury.

In conclusion, our data suggest that amlodipine could provide a beneficial effect against TI lesions caused by hyperoxaluria and therefore preserve renal function, probably by a nonhemodynamic pathway.

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The authors gratefully acknowledge the valuable contribution of Carolina Mourele, who helped in the preparation of laboratory experiments, and Heriberto Engel, MD, who thoroughly reviewed the style of this manuscript.

\section*{References}
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