Temporal Changes in mRNA Expression for Bikunin in the Kidneys of Rats during Calcium Oxalate Nephrolithiasis

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Abstract. Inter-α-inhibitor and other bikunin-containing proteins are synthesized in relatively large quantities by the liver. These proteins function as Kunitz-type serine protease inhibitors and appear capable of inhibiting calcium oxalate (CaOx) crystallization in vitro. Preliminary studies have shown that renal tubular epithelial cells synthesize bikunin in response to CaOx challenge. To examine this response in vivo, a sensitive reverse transcription-quantitative competitive template-PCR was developed to detect and quantify poly(A)⁺-tailed bikunin mRNA expression in kidney tissue from normal rats and rats developing CaOx nephrolithiasis after challenge with ethylene glycol. Bikunin mRNA expression in rat liver tissue was assessed as a positive control. The expression of bikunin mRNA in liver did not differ significantly between normal control rats and experimental rats with induced hyperoxaluria and renal CaOx crystallization. In contrast, there were significant temporal increases in the levels of bikunin mRNA expression in rat kidneys during CaOx nephrolithiasis after challenge with ethylene glycol. Urinary excretion of bikunin-containing proteins seemed to increase concomitantly. These findings indicate an association between the induction of hyperoxaluria/CaOx nephrolithiasis and the expression of the bikunin gene in rat kidneys.

Bikunin is a small, 35- to 40-kD polypeptide that is synthesized in hepatocytes as the carboxyl end of a precursor molecule containing α₁-microglobulin (1–3). The bikunin moiety may exist as a single glycoprotein or may be linked covalently (via a chondroitin sulfate bridge) to one or more polypeptide chains, referred to as heavy chain-1 (HC-1), HC-2, and HC-3 (4–7). Combinations of these chains give rise to at least two molecules, i.e., inter-α-inhibitor (IαI or ITI), composed of bikunin, HC-1, and HC-2, and pre-inter-α-inhibitor (PαI), composed of bikunin and HC-3 (7). IαI, with a molecular mass of approximately 220 kD, and PαI, with a molecular mass of approximately 150 kD, are Kunitz-type serine protease inhibitors that react with trypsin, chymotrypsin, cathepsin, elastase, plasmin, and acrosin (8–11).

Free bikunin is present in both the plasma and urine of human subjects and rodents (12–14). The origin of plasma and urinary bikunin is thought to be the liver; this concept is supported by the studies of Lindqvist et al. (2) and Salier et al. (15), which showed that mRNA coding for bikunin- or IαI-related proteins are expressed in liver but not other organs. Although the functions of bikunin remain unknown, in vitro studies have shown that urinary bikunin can act as an inhibitor of calcium oxalate (CaOx) crystallization (12,14,16–18).

Recently, we observed increased expression of bikunin mRNA in renal epithelial cells exposed to oxalate and CaOx crystals (19) and increased urinary excretion of IαI-related proteins by rats with hyperoxaluria and CaOx nephrolithiasis (S. Iida, A. B. Peck, K. J. Byer, and S. R. Khan, unpublished results). These observations suggested that hyperoxaluria and subsequent CaOx crystal deposition in the kidney might induce increased synthesis of this family of proteins. On the basis of the studies by Kastern et al. (20), which suggested that α₁-microglobulin mRNA is expressed in the kidney, we have investigated whether the increased urinary levels of IαI-related proteins might be attributable to a direct response by the kidney itself. Thus, in this study, we have used a sensitive quantitative competitive (QC) DNA template-PCR system to examine the expression of bikunin mRNA in rat kidneys during induction of hyperoxaluria, with subsequent deposition of CaOx crystals in the renal tubules.

Materials and Methods

Animals

Male Sprague Dawley rats weighing 70 to 80 g were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed in the Department of Animal Resources at the University of Florida. The rats were housed individually in metabolic cages and given free access to water and food. Urine specimens were collected daily in ice-cooled 50-ml conical centrifuge tubes containing 0.02% sodium azide.
Induction of Hyperoxaluria and Tissue Preparation

Hyperoxaluria, with subsequent renal CaOx crystal formation, was induced in experimental rats according to the protocol described by Khan (21). In brief, experimental rats \( (n = 10) \) received water containing ethylene glycol (EG) \((0.75\%\, \text{vol/vol}) \) for either a 2-wk \( (n = 5) \) or 8-wk \( (n = 5) \) period. Control rats \( (n = 10) \) received normal water. At the appropriate times, rats were euthanized and their livers and kidneys were removed. The livers and left kidneys were stored in liquid nitrogen for molecular biologic studies, whereas the right kidneys were fixed in 10% phosphate-buffered formalin for histoch- emical studies. A central slice of each formalin-fixed right kidney was embedded in paraffin. Sections were stained with hematoxylin and eosin and examined with bright-field (to view tissue histologic features) or polarized-light (to view crystal deposition) optics.

For immunocytochemical staining, kidney sections were depara- finized, incubated with 3% hydrogen peroxide/phosphate-buffered saline (PBS) for 20 min, washed with PBS, and then incubated with 3% bovine serum albumin for 60 min. Each section was incubated overnight at 4°C with rabbit anti-human inter- a trypsin inhibitor (ITI) antibody (Accurate Chemical & Scientific Corp., Westbury, NY) diluted 1:50. Sections were then rinsed with cold PBS and incubated with goat anti-rabbit IgG (H + L) human/mouse horseradish peroxi- dase for 60 min at room temperature. Sections were rinsed, treated with 3,3’-diaminobenzidine, counterstained with hematoxylin, and examined using a light microscope.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Urinary Proteins

Individual rat urine samples were collected for 24 h and then adjusted to equal volumes. Five microliters of each sample were subjected to electrophoresis through 10 to 20% gradient gels, using the Laemmli buffer system and a Bio-Rad Mini-Protean II apparatus (Bio-Rad Laboratories, Hercules, CA). Prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) low-range standards (Bio-Rad Laboratories) were included on each gel. Proteins were observed in the gels by using Coomassie Brilliant Blue R-250 or silver staining (Bio-Rad Laboratories).

Western Blotting and Immunocytochemical Staining

After SDS-PAGE, unstained gels were transferred electrophoretically (50 V for 2 h) to BioBlot nitrocellulose membranes (Coster Scientific Co., Cambridge, MA), using a Mini-Transblot apparatus (Bio-Rad Laboratories). The nitrocellulose membranes were blocked with milk (5%) in Tris-buffered saline/Tween (6.38 mM Tris-HCl, 25 mM NaCl, 0.05% Tween 20, pH 8.0). Membranes were treated for 16 h with rabbit anti-human IaI antibody (Dako, Carpinteria, CA) diluted 1:1000 in Tris-buffered saline/Tween with 5% milk. Membranes were then treated for 2 h with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Bio-Rad Laboratories) diluted 1:5000. Bound antibody was detected through chromogenic development (enzyme-linked immunosor- bents assays) using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solutions (Boehringer Mannheim, Indianapolis, IN).

Reverse Transcription and QC-PCR

Poly(A)+-tailed mRNA was isolated from rat tissues (50 mg) using Oligotex Direct mRNA mini kits (Qiagen, Santa Clarita, CA), according to the protocol provided by the manufacturer, and was then converted to cDNA with reverse transcriptase. In brief, 40-μl reactions contained 14 μl of mRNA template, 4 μl of random primers (Pharmacia, Piscataway, NJ), 8 μl of 5× PCR buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 2 μl of dNTP mixture (10 mM), 2.7 μl of dithiothreitol (0.1 M), 1.3 μl of RNAsin (52 U) (Promega, Madison, WI), 4 μl of acetylated bovine serum albumin, and 4 μl of Superscript II reverse transcriptase (200 U) (Life Technologies, Gaithersburg, MD). The mRNA template and random primers were mixed and preincubated for 5 min at 65°C before being placed on ice for 1 min. The reverse transcription reaction was performed for 60 min at 42°C and then stopped by heating to 95°C for 10 min.

PCR reactions were performed according to protocols detailed elsewhere (22). Amplifications were performed as 25-μl reactions consisting of 1 μg of cDNA, 0.25 μl of recombinant Taq DNA polymerase (0.65 U) (Qiagen), 2.5 μl of 10× reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂), 200 μM dNTP mixture, and 1.25 μl each of the 5’- and 3’-primers (20 μM). Quantitative PCR contained 1 μl of a dilution (10² to 10⁹ copies) of synthetic DNA template. After an initial 3-min denaturation step, PCR consisted of 35 cycles, where each cycle consisted of 45 s of denaturation at 94°C, 1.5 min of annealing at 57°C, and 1 min of extension

Figure 1. Scheme used for the development of quantitative competitive (QC)-PCR. The competitive template was synthesized to contain the 5’ and 3’ primers for the normal bikunin gene but only 312 bp of the open reading frame sequence (A). PCR amplification of the competitive DNA template results in a 312-bp product, using the specific bikunin primer pair, and the number of amplicons can be enumerated using serial dilutions (1:10¹ to 1:10⁹) of the competitive template. Quantification of the PCR product(s) is accomplished using calibration curves for the logarithm of the DNA template intensity (measured using an image analyzer) (B).
at 72°C. The primer sequences for bikunin were as follows: sense primer, 5'-GCAGTGCTGCCCCAAGAG-3', corresponding to nucleotides 633 to 650 of rat bikunin mRNA; antisense primer, 5'-ACTGCGTGTTAGCTCCTCGTA-3', corresponding to nucleotides 1047 to 1067 (2). To control for the presence of different amounts of cDNA in different mRNA preparations, cyclophilin mRNA was amplified from each cDNA. Primer sequences for cyclophilin were as follows: sense primer, 5'-TTTATGTGTCAGGGTGGTGACTTCA-3', corresponding to nucleotides 192 to 216; antisense primer, 5'-TATTCATGCCTTCTTTTCAGTACTTGCA-3', corresponding to nucleotides 403 to 426 (23).

Quantitative PCR was performed using an internal competitive template, as described elsewhere (24). In brief, this QC-PCR is based on the assumption that the cDNA template and the competitive template compete equally for the primers and that amplification is colinear. To develop a competitive DNA template for use as an internal control, a 312-bp fragment of the bikunin gene flanked by sequences homologous for the 5' and 3' ends and containing the gene-specific probe sites was synthesized (Figure 1). To accomplish this, a PCR was performed with the 5' primer plus a modification of the 3' primer. The modified 3' primer (5'-ACTGCGTGTTAGCTCCTCACCAGGATCCGCAAGGC-3'), corresponding to nucleotides 909 to 926 and 1047 to 1067 of rat bikunin mRNA (2), consisted of two portions, i.e., a 5' end that annealed to the normal binding site of the 3' end of the bikunin gene, leaving the 3' end of the primer unannealed, and a 3' end that annealed at an internal site, leaving the 5' end of the primer unannealed.

PCR using this primer pair preferentially amplified the shorter 294-bp segment and synthesized the 3' end primer site, resulting in a 312-bp product. This PCR fragment was size-fractionated by electrophoresis through a 1.3% agarose gel; the 312-bp band was isolated using the Qiagen DNA extraction protocol (Qiagen) and was ligated into the TA cloning vector pCR 2.1 (Invitrogen, San Diego, CA). Recombinant pCR2.1 plasmids were selected after transformation of and growth in INV®-competent cells. Positive colonies were confirmed by identification and sequencing of a proper insert fragment, after digestion with EcoRI. The plasmid DNA was purified by alkaline lysis and polyethylene glycol precipitation (25) and was then quantified. QC-PCR was performed using 1 μl of serial dilutions of the quantitative template to establish standard curves (Figure 1).

Detection and Quantitation of PCR Products

PCR products were resolved by electrophoresis through 1.3% agarose gels containing ethidium bromide and were then observed with ultraviolet light. The gels were scanned and the intensity of each PCR fragment was measured using an image analyzer (Eagle Eye II; Stratagene, La Jolla, CA). Band intensities were normalized for dif-
ferences in molecular weights. Target message was quantified by determining where the logarithmic ratios of template and target DNA band intensities were equal (24). Samples were analyzed three times by PCR, and the results were expressed as the average of the three values. To confirm that the PCR bands corresponded to bikunin mRNA, appropriate bands were isolated and sequenced.

**Statistical Analyses**

Data are expressed as mean ± SD. Differences between data sets were analyzed by the Mann–Whitney test. A level of $P < 0.01$ was considered statistically significant.

**Results**

**Induction of CaOx Nephrolithiasis**

Rats receiving drinking water containing EG exhibit increased urinary excretion of oxalate, with subsequent formation of CaOx crystals that are deposited in renal tubules (21). Histologic examination revealed that rats receiving EG through their drinking water deposited CaOx crystals, whereas kidneys of control rats contained no crystal deposits (Figure 2). After 2 wk of treatment with EG, occasional CaOx crystals were observed and tubular cells appeared normal (Figure 2A). By 8
wk of treatment, CaOx microliths were abundant in the kidney tubules and epithelial cell damage was obvious (Figure 2B).

Increased Urinary Excretion of Bikunin in Nephrolithic Rats

Aliquots of 24-h urine samples collected from untreated and EG-treated rats at 0, 2, or 8 wk were subjected to SDS-PAGE. The separated urinary proteins were transblotted onto nitrocellulose filters, the filters were treated with rabbit anti-human ITI antibody, and the bound antibody was detected by enzyme-linked immunosorbent assay. Rabbit anti-human ITI antibody has been shown to recognize as many as seven separate protein moieties in urine, i.e., whole IαI (240 kD), PαI (120 kD), free HC-3, HC-2, and HC-1 (55 to 80 kD), bikunin (40 kD), and an unknown moiety (20 kD) that is possibly a degradation product of bikunin (26). In urine from untreated rats, IαI and PαI either were totally absent or were present in amounts generally below detection, whereas bikunin, the three HC proteins, and the 20-kD moiety were easily detected (Figure 3A). Of note, excretion of bikunin and the three HC proteins increased in untreated rats as the rats aged. In contrast, the urine of EG-treated rats showed not only the expected age-related increase in the levels of bikunin and the HC proteins but also the appearance of IαI and PαI, the levels of both of which increased over the 8-wk treatment period. Quantitative comparisons of selected protein bands in urine from untreated and EG-treated rats indicated that IαI levels were increased in EG-treated rats but bikunin and HC-2 levels were not (Figure 3B). Therefore, consumption of EG resulted in quantitative as well as qualitative differences in urinary excretion of IαI-related proteins.

Detection of Bikunin mRNA Transcripts in Normal Rat Kidney

To determine whether changes in the urinary excretion of IαI-related proteins by EG-treated rats resulted from stimulation of the kidney or liver, cDNA prepared from liver and kidney tissue was used as a template in PCR. As shown in Figure 4 (top), bikunin mRNA was detected in both normal and experimental kidneys as a single band of 434 bp, although the PCR bands from normal kidneys were less intense than those from the EG-stimulated kidneys. High levels of expression of bikunin mRNA were seen in the liver, but expression was not detected in either heart myocytes or peripheral blood leukocytes. PCR bands for cyclophilin (Figure 4, bottom) showed similar band intensities in different cDNA preparations.

To ensure that the PCR products of these amplifications were bikunin, each PCR band of approximately 440 bp was extracted from the gels and ligated into the TA cloning vector pCR2.1. Recombinant plasmids were selected, and the ligated inserts were sequenced. The rat kidney and liver bikunin cDNA sequences were then compared with the published sequences of both mouse and rat liver bikunin. As presented in Figure 5, the DNA sequence of Sprague Dawley rat kidney bikunin contained only a 1-bp difference from the published sequence of Wistar or Sprague Dawley rat liver bikunin but showed 33-bp differences from mouse liver bikunin. Therefore, bikunin mRNA is detectable in the rat kidney.

Increased Synthesis of Bikunin mRNA Transcripts in Nephrolithic Rat Kidneys

cDNA prepared from rat liver and kidney tissues were used as templates in QC-PCR, to determine the actual levels of mRNA transcripts encoding the open reading frame of bikunin. As shown in Figure 6A (left), bikunin cDNA could be amplified from liver tissue in the presence of control template DNA diluted >10²-fold. In contrast, control template DNA needed to be diluted >10⁶-fold before bikunin cDNA could be amplified from an equal amount of kidney tissue (Figure 6A, right). On the basis of these data, approximately 10⁵ bikunin mRNA transcripts/μg tissue were present in liver, whereas only approximately 10³ bikunin mRNA transcripts/μg tissue were present in kidney (Figure 6B). To quantify changes in kidney

**Figure 4.** Detection of bikunin mRNA transcripts in kidney cells of both control and EG-treated rats. Poly(A)+-tailed mRNA was isolated from a number of rat tissues and converted to cDNA using random primers plus reverse transcriptase, and the cDNA (1 μg cDNA/reaction) was used as a template for each PCR, for detection of bikunin (top) or cyclophilin (bottom). The PCR products were subjected to electrophoresis through 1.3% agarose gels. Bikunin and cyclophilin amplicons were detected as single bands of 434 and 235 bp, respectively.
bikunin mRNA expression, QC-PCR was performed using samples from rats that had been treated with EG for 2 or 8 wk. As shown in Figure 7, a 2-wk exposure to EG resulted in an approximately twofold increase in the expression of kidney bikunin (630 versus 1200 bikunin mRNA transcripts/mg cDNA), whereas an 8-wk exposure to EG resulted in an approximately 17-fold increase (10,000 bikunin transcripts/mg cDNA). Statistical analysis of the data revealed that the increased expression of bikunin mRNA transcripts in the kidney during the 8-wk treatment period was highly significant (P < 0.01); however, there were no significant differences in the expression of liver bikunin mRNA transcripts between the normal and EG-treated groups with time (Table 1). Therefore, the effect appears to be focused primarily on the kidneys.

Increased Synthesis of IαI Protein by Kidney Cells in Response to Hyperoxaluria

Anti-IαI antibody was used for immunocytochemical staining of kidney sections before and after the induction of hyperoxaluria. In kidneys from untreated rats, staining was limited primarily to the proximal tubules and generally to the luminal contents (Figure 8A). Two weeks after initiation of the hyperoxaluric protocol, all sections of the nephron, including both proximal and distal tubules, stained positively for IαI (Figure 8B). Positive staining was observed in both the luminal contents and the cells. Crystal-associated organic material also stained positively. Staining was even more intense and uniform after 4 wk of hyperoxaluria (data not shown).

Discussion

Proteins are the predominant macromolecules in the urine and are often the major components of urinary stone matrices (27). Many of these proteins have been shown in vitro to influence, both positively and negatively, the formation of crystals in urine, including the formation of CaOx crystals. Nephrolithiasis involves several steps, including crystal nucle-
ation, growth, aggregation, and retention within the kidneys, all of which are thought to be modulated by urinary proteins. Some of these proteins, such as Tamm-Horsfall protein (28) and osteopontin (29–32), are produced by renal epithelial cells, whereas others, such as prothrombin-fragment-1 and IαI, originate in the liver. However, there are reports (20,33) suggesting that urinary prothrombin fragment-1 and IαI may be produced by kidney cells as well. Results presented here demonstrate that bikunin mRNA is synthesized in both liver and kidney tissue and that kidney cells are stimulated to increase bikunin mRNA synthesis during the development of CaOx urolithiasis.

Bikunin was originally identified as uronic acid-rich protein, on the basis of its high uronic acid content (12). Amino-terminal amino acid sequence analysis of both rat and human uronic acid-rich protein revealed homology with bikunin (14,15). More recently, Tang et al. (13) reported the presence in human urine of two isotypes of bikunin, a 35-kD protein and a 21-kD protein (HI-14), that inhibited CaOx crystal formation. Atmani et al. (34) identified a third isotype of bikunin, a 45-kD protein. These studies did not identify the source of the molecular species, although it was speculated that only one isoform originated in the liver and the other species were formed after exiting the liver.

In this study, we used a sensitive and quantitative PCR method to demonstrate that (1) both rat liver and kidney tissues express bikunin mRNA transcripts; (2) expression of bikunin mRNA transcripts/1 μg cDNA are present in liver but only 10^3 bikunin mRNA transcripts/1 μg cDNA are present in kidney tissue.

Figure 6. Comparison of the quantification of bikunin mRNA transcripts in normal control rat liver and kidney cells. (A) Bikunin cDNA was amplified from either liver (left) or kidney (right) tissues in the presence of the competitive DNA template (QCT) diluted >10^4-fold. (B) Quantification of the amplicons indicated that approximately 10^9 bikunin mRNA transcripts/1 μg cDNA are present in liver but only 10^3 bikunin mRNA transcripts/1 μg cDNA are present in kidney tissue.
mRNA transcripts increases in kidney tissue in response to EG-induced hyperoxaluria and CaOx nephrolithiasis, and urinary excretion of high-molecular weight, bikunin-containing members of the IαI family of proteins is increased in response to oxalate and CaOx crystals. These results agree with the suggestions of Kastern et al. (20) and Itoh et al. (35) that mRNA coding for α1-microglobulin/bikunin precursors can be expressed in the kidneys. In addition, the results of these studies using rats confirm those of Marengo et al. (36), which showed that levels of both high molecular weight, bikunin-containing proteins (IαI and PαI) are increased in CaOx kidney stone-forming men, compared with gender-matched control subjects or stone-forming female patients.

Hyperoxaluria and CaOx nephrolithiasis are known to stimulate renal epithelial cells to produce a variety of macromolecules. For example, rats with hyperoxaluria and CaOx crystal deposition exhibit increased immunocytochemical staining in kidneys for both Tamm-Horsfall protein and osteopontin (37). Furthermore, exposure of renal epithelial cells in culture to CaOx crystals induces the expression of mRNA transcripts for

Figure 7. Increased synthesis of bikunin mRNA transcripts in rat kidneys during development of nephrolithiasis. (A) QC-PCR of bikunin mRNA transcripts present in kidney tissues of rats that had been treated with EG for 2 wk (left) or 8 wk (right). (B) Quantitative analysis, indicating an increase in the expression of kidney bikunin of approximately twofold after 2 wk of exposure to EG and 17-fold after 8 wk of exposure, compared with untreated rats.
c-Myc, early growth response protein-1, and nuclear receptor-77 (38), whereas 2- to 6-h exposure stimulates the expression of genes encoding plasminogen activator and platelet-derived growth factor A-chain. Such exposures produce enhanced expression of osteopontin mRNA and protein secretion into the medium (39).

Quantitative analyses of Western blots indicated that urinary excretion of free bikunin did not change, whereas excretion of bikunin-containing IαI-related proteins was increased. In addition, there was an increase in immunocytochemical staining of kidneys for IαI-related proteins, and both the cells and luminal surfaces of renal tubules were stained. It is, however, difficult to establish the origin of these proteins in the urine of our study animals, because neither the GFR nor bikunin concentrations in the urine or plasma were measured. Because there was no significant increase in the expression of bikunin mRNA transcripts in the liver after EG treatment, we suggest that any increase in the urinary excretion of bikunin-containing IαI-related proteins would not be derived from increased production in the liver. It is well known that undegraded bikunin can be filtered at the glomeruli and that the urinary concentration of bikunin is dependent on the GFR (40,41) and is significantly increased in urine after renal dysfunction (42). Our earlier studies showed that in the animal model of CaOx nephrolithiasis used here, kidney function is not severely impaired (43), although Okada et al. (44) reported a loss of renal function in rats receiving EG and vitamin D. Therefore, increased urinary excretion of bikunin-containing proteins in our

Table 1. Relative expression levels of bikunin mRNA in rat liver and kidneys

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time of Testing</th>
<th>n</th>
<th>Control</th>
<th>Hyperoxaluric Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2 wk</td>
<td>5</td>
<td>176310 ± 85470.5</td>
<td>170074 ± 84775.8</td>
</tr>
<tr>
<td></td>
<td>8 wk</td>
<td>5</td>
<td>189528 ± 86658.5</td>
<td>173147 ± 71250.3</td>
</tr>
<tr>
<td>Kidneys</td>
<td>2 wk</td>
<td>5</td>
<td>710 ± 166.4</td>
<td>1240 ± 415.9</td>
</tr>
<tr>
<td></td>
<td>8 wk</td>
<td>5</td>
<td>776 ± 147.7</td>
<td>10260 ± 698.6b</td>
</tr>
</tbody>
</table>

Results are given as mean ± SD.

Liver: control versus hyperoxaluric rats: NS.
2 wk versus 8 wk rats: NS.
Kidneys: 2 wk control versus hyperoxaluric rats: NS.
8 wk control versus hyperoxaluric rats: P <0.01.

Figure 8. Immunolocalization of IαI-associated proteins in the renal cortex of untreated rats (A) or EG-treated rats (B) at 2 wk. Anti-IαI antibody staining of renal sections from untreated rats is primarily limited to the luminal contents of the tubules. Staining of renal sections from EG-treated rats occurs within the cells and tubular lumina of all sections. Magnification, ×800.
study animals might be a result of reductions in renal function, and the increased immunocytochemical staining in renal tubules might reflect increased uptake of proteins by renal epithelial cells.

Our studies noted only a twofold increase in the expression of bikunin mRNA in kidneys after a 2-wk treatment with EG but revealed a 17-fold increase after an 8-wk treatment. During the early stages, renal epithelial cells exhibited limited changes, probably because of exposure to increased oxalate levels. By 8 wk, however, crystal deposition and renal cell changes, probably because of exposure to increased oxalate levels. By 8 wk, however, crystal deposition and renal cell changes were clearly visible. Therefore, the early and limited increase in the levels of bikunin mRNA transcripts is primarily a result of exposure to oxalate ions, whereas the latter and marked increase is associated with the deposition of CaOx crystals. Additional studies are currently under way to determine the origin of bikunin and other I1D-related proteins in the urine and to elucidate the mechanisms whereby hyperoxaluria and CaOx crystal deposition stimulate renal epithelial cells.

Interactions between renal epithelial cells and CaOx crystals (or oxalate ions) no doubt play a crucial role in the formation of urinary stones (20,45). Renal epithelial cells respond to hyperoxaluria and the presence of CaOx crystals both in vivo (37) and in vitro (38,39). Moderate hyperoxaluria and/or occasional crystals may provoke protective responses and cause the renal epithelial cells to increase the synthesis and secretion of nephrolithiasis modulators. Calcium-binding proteins coat calcium and coat the surfaces of calcific crystals, which are then either excreted as crystalluria particles or endocytosed by the epithelial cells. Crystals produced in such an environment have protein coats and apparently are less reactive with renal epithelial cells.

Prolonged hyperoxaluria and exposure to CaOx crystals, as in this study, can induce inflammation and injury to the epithelial layer. Proteins produced by injured cells might be abnormal and less reactive with crystal surfaces. If they are, then the exposed crystal surfaces might be considerably more injurious to renal cells, resulting in degradation and sloughed basal lamina. Membranous cellular degradation products are known to promote CaOx crystallization by heterogeneous nucleation at very low supersaturation, and crystals adhere to exposed basement membrane. Kidney stone formation has generally been considered a physicochemical phenomenon. The results of this study indicate that the pathogenesis of stone formation may be similar to other pathologic biominerization processes in which inflammation plays a significant role.

Acknowledgments

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