Heparan Sulfate (HS)/Heparan Sulfate Proteoglycan (HSPG) and Bikunin are Up-regulated During Calcium Oxalate Nephrolithiasis in Rat Kidney

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Summary: We reported that expression of both HSPG and of bikunin are increased in calcium oxalate (CaOx) nephrolithic rat kidneys (Iida et al., *J. Am. Soc. Nephrol.* 1999, *Urol. Res.* 1997). However, these findings were obtained from separate experiments. The present study evaluates whether levels of HSPG and bikunin expression differ in the rat kidney during calcium oxalate nephrolithiasis. Twenty-four male Wistar rats weighing 200-250 g were assigned to one of four groups (n=6 each group) and administered with 0.5% ethylene glycol daily and 0.5 μ g of 1 α -OH-D₃ every other day to induce CaOx nephrolithiasis. Animals were sacrificed 1 or 2 weeks later and both kidneys were excised. The cortex was separated from the medulla and papillary tips in the right kidney, then stored in liquid nitrogen for quantitative competitive-reverse transcription-polymerase chain reaction (QC-RT-PCR). The left kidney was fixed in 10% buffered formalin for histochemical studies. We assessed the variable gene expression of both HSPG and bikunin by QC-RT-PCR. Immunohistochemical analyses of left kidney tissue samples determined the localization of HSPG and bikunin. Normal rats serving as controls (n=6 each) were also sacrificed and processed in the same manner as the experimental groups. QC-RT-PCR confirmed that HSPG and bikunin mRNA expression is significantly increased in nephrolithic kidneys ($p < 0.05$; Mann-Whitney test), and that medulla and papillary tips tended to express more mRNA of both. Immunohistochemical studies revealed that the production of HS and bikunin was increased in both the distal and proximal tubules of nephrolithic kidneys. These findings suggest that the increased expression of both HSPG and bikunin play an important role during calcium oxalate stone formation. In addition, this phenomenon might be associated with the progression of urothelial damage.

Key words heparan sulfate (HS), heparan sulfate proteoglycan (HSPG), bikunin, calcium oxalate (CaOx), nephrolithiasis

INTRODUCTION

The potential role of urinary proteins in stone pathogenesis has become a key issue in studies of urolithiasis. Urinary proteins must fulfil some controlling function in stone pathogenesis as promoters or inhibitors of calcium oxalate (CaOx) crystallization. Indeed, several human urinary proteins may alter the nucleation, growth or aggregation of CaOx crystals. This notion combined with advances in protein

chemistry has led to many investigations into the physicochemical processes of stone formation. [1,2]. Among the known urinary macromolecular substances including Tamm-Horsfall glycoprotein [3], uronic acid rich protein (UAP) [4] and urinary prothrombin fragment 1 [5], nephrocalcin may be the principal inhibitor in urine [6] since it accounts for approximately 90% of the total inhibitory effect of urine on CaOx crystallization [6-8]. Heparan sulfate (HS) is a major glycosaminoglycan (GAG) compo-

ment of the matrix of CaOx stones and is also a potent inhibitor of CaOx crystallization [9,10]. On the other hand, bikunin is the light chain of inter- α -trypsin inhibitor (ITI or I α I) and also potently inhibits CaOx crystallization. [11-14]. The results of previous studies suggest that the increased expression of both HSPG and bikunin mRNAs in rat kidney correlates with CaOx crystal formation, deposition in the urothelial cells and cell injury [15-17]. However, these findings were obtained from separate experiments. We developed a quantitative competitive reverse transcription-PCR (QC-RT-PCR) method with which to investigate the mRNA expression of macromolecules in the kidneys [16-18]. This method can now determine exact transcript numbers of target messages. We performed QC-PCR in the present study to measure expression levels of HSPG and bikunin mRNA during CaOx stone formation in the rat renal medulla and cortex. In addition, we localized HS and bikunin/ITI-related proteins in the rat kidney using immunohistochemical techniques.

MATERIALS AND METHODS

Animals

Twelve male Wistar rats weighing about 200 g were divided into two equal groups, which were administered 0.5% ethylene glycol daily and 0.5 μ g of 1- α -OH-D₃ every other day by gavage [19]. The animals were sacrificed after 1 or 2 weeks (wks) and both kidneys were removed. The right kidney was stored in liquid nitrogen and the left was fixed in 10% formalin for histochemical studies. Twelve normal control rats were also divided into two groups of six rats each, were sacrificed after 1 or 2 wks, and their kidneys were processed in the same manner.

Reverse transcriptase and quantitative competitive PCR

Poly(A)+ tailed mRNA was isolated from rat livers and kidneys (50 mg) using Quick Prep Micro mRNA Purification Kits (Pharmacia Biotech, Milwaukee, WI), then converted to cDNA with reverse transcriptase. In brief, 20 μ l reactions contained 12 μ l of mRNA template, 2 μ l of 10 $\times$ RT buffer, 2 μ l of dNTP mix (10 mM), 2 μ l of random primer (GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD), 1 μ l of RNase inhibitor (10 units/ μ l, Promega, Madison, WI), and 1 μ l of Omniscript reverse transcriptase (Qiagen GmbH, Germany). The RT reaction proceeded for 1 h at 37 $^{\circ}$ C, and was

terminated by heating for 5 min at 93 $^{\circ}$ C. Samples were PCR amplified as 25 μ l reactions consisting of 1 μ g of cDNA, recombinant Taq DNA polymerase (0.65 U) (Qiagen GmbH, Germany), 2.5 μ l of 10 $\times$ buffer (10 mM Tris-HCl, [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂), 200 μ M deoxyribonucleotide triphosphates (dNTPs) and 1.25 μ l each of the 5' and 3' primers (20 μ M each). Primers for PCR amplification of bikunin were based on sequences reported for the rat HSPG [20] and bikunin genes [21] (Table 1). Variability in the mRNA content was assessed by simultaneously amplifying cyclophilin mRNA [22] because it is expressed in virtually all types of tissues and seems to have been highly conserved throughout mammalian evolution [23].

The PCR reaction mixture (25 μ l) included 1 μ g of the cDNA product, 0.13 μ l of recombinant Taq DNA polymerase (QIAGEN GmbH, Germany), 2.5 μ l of 10 $\times$ PCR buffer (Qiagen), 0.5 μ l of dNTP mixture, and 1.25 μ l each of the 5' and 3' primers (20 μ M). Cyclophilin was amplified by 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 55 $^{\circ}$ C for 40 s, and extension at 72 $^{\circ}$ C for 90 s. HSPG was amplified by 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 60 $^{\circ}$ C for 60 s and extension at 72 $^{\circ}$ C for 90 s. Bikunin was amplified by 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 57 $^{\circ}$ C for 90 s, and extension at 72 $^{\circ}$ C for 60 s, using a Thermal Cycler MP (Takara Biomedicals, Shiga, Japan).

QC-PCR was performed using each of the QC-templates as described elsewhere [16,17]. This QC-PCR is based on the assumptions that the cDNA template (10¹-10⁹ copies) and an appropriate competitive template will compete equally for the primers and that amplification will be co-linear. To develop competitive DNA templates for use as an internal control, a 312 bp fragment of the bikunin gene and a 345 bp fragment of the HSPG gene flanked by sequences homologous for 5' and 3' ends and containing the gene-specific probe sites were synthesized [16,17] using PCR with the 5'-primer and a modified 3'-primer (Table 1). The latter consisted of two portions: a 5'-end that annealed to the normal binding site of the 3'-end of the target gene leaving the 3'-end of the primer free, plus a 3'-end that annealed at an internal site leaving the 5'-end of the primer free. PCR using this primer pair preferentially amplified the shorter DNA fragment containing the 3'-end primer site. This PCR fragment was size-fractionated by electrophoresis through 1.3% low-melting agarose gels (FMC Bioproducts, Rockland, ME), then the band was isolated using QIAquick DNA extraction

TABLE 1.
Primer sequences

	Sequence	Corresponding uncletides
HSPG *		
5'-primer	5'-TGAAGAAGAAGGACGAAGGC-3'	(1346-1365)
3'-primer	5'-CCGGATGATGTTGAATTGG-3'	(1851-1833)
Modified	5'-CCGGATGATGTTGAATTGG-	
3'-primer	GAGGGAATGGGCCTAGAGAA-3'	(1651-1670/1851-1833)
Bikunin **		
5'-primer	5'-GCAGTGCTGCCCCAAGAG-3'	(633-650)
3'-primer	5'-ACTGCGTGTTAGCTCCTCGTA-3'	(1047-1067)
Modified	5'-ACTGCGTGTTAGCTCCTCGTA-	
3'-primer	CCAGAGTTCTGCAAAGGC-3'	(909-926/1047-1067)
Cyclophilin [†]		
5'-primer	5'-TTTATGTGTCAGGGTGGTGACTTCA-3'	(192-216)
3'-primer	5'-TATTCATGCCTTCTTCACTTTGC-3'	(403-426)

GenBank Accession Number: * U73184, ** S87544, [†] Y00052

protocol (Qiagen, Inc.) and ligated into the TA cloning vector, pCR2.1 (Invitrogen, Inc, San Diego, CA). Recombinant pCR2.1 plasmids were selected following transformation of and growth in INV α F' competent cells. Positive colonies were confirmed by the identification and sequencing of a proper insert fragment following *Eco*RI digestion. Plasmid DNA was purified by using a QIAfilter Plasmid Maxi kit (Qiagen) and quantified. QC-PCR was carried out using 1 μ l serial dilutions of the QC-template to establish standard curves (data not shown).

Detection and quantification of PCR products

PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. The intensity and area of each fragment were measured using an image analyzer (Gel Doc 1000, Bio-Rad, Hercules, CA). Band intensities were normalized for differences in molecular weights. Target message was quantified by determining where the log ratios of template and target DNA band intensities were equal [24]. We also sequenced and compared the PCR products with those of cDNA clones.

Histochemical study

The left kidneys of rats from the control and experimental groups were processed for light microscopic examination. Paraffin sections were stained with hematoxylin-eosin. Some of the sections were examined immunohistochemically as follows. In brief, paraffin sections were deparaffinized, then

incubated with phosphate-buffered saline (PBS), pH 7.4, for 5 min, followed by 0.1% trypsin at 37 °C for 3 hrs. The sections were washed 3 times with PBS for 5 min each, then placed in blocking buffer for 30 min at room temperature. Each section was rinsed with PBS and incubated overnight at 4 °C with anti-mouse IgM HS monoclonal antibody (HepSS-1; Seikagaku Kogyo, Tokyo, Japan) diluted 1:1000. The sections were rinsed with cold PBS and incubated with biotinylated anti-mouse IgM antibody for 30 min at room temperature. The sections were rinsed again with PBS, then immersed in diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA), counterstained with methyl green and examined using a light microscope. We used rabbit anti-human ITI polyclonal antibody (Dako, Carpinteria, CA) to immunohistochemically localize bikunin and I α I-related proteins [17]. In brief, the sections were deparaffinized, incubated with 3% hydrogen peroxide/phosphate-buffered saline (PBS) for 20 min, washed with PBS, then incubated with 3% bovine serum albumin for 60 min. Each section was incubated overnight at 4 °C with rabbit anti-human inter- α -trypsin inhibitor (ITI) polyclonal antibody (Accurate Chemical & Scientific Corp., Westbury, NY) diluted 1:50. Sections were rinsed, immersed in 3,3'-diaminobenzidine, counterstained with hematoxylin, and examined using a light microscope.

Statistical analyses

Data are expressed as means $\pm$ S.E. Differences between data sets were analyzed using the Mann-

Whitney test. Values of $p < 0.05$ was considered statistically significant.

RESULTS

RT-PCR analysis

Cyclophilin, bikunin and HSPG mRNA were expressed in both control and experimental rat kidneys as individual bands of 235, 434 and 506 bp, respectively. The intensity of cyclophilin expression was equal between control and experimental groups. On the other hand, more bikunin and HSPG mRNA

was expressed in nephrolithic than in normal kidneys (Fig. 1).

Increased synthesis of HSPG and bikunin mRNA transcripts in nephrolithic rat kidney

To quantify changes in both HSPG and bikunin mRNA transcripts, we performed QC-PCR upon cDNA samples obtained from CaOx stones from rat kidneys and from normal kidney tissues. Figure 2 (left panel) shows that HSPG cDNA was amplified from the normal rat kidney (medulla, 2wks) in the presence of QC-template DNA diluted more than 1×10^8 . In contrast, the QC-DNA template had to be

Fig. 1. Detection of heparan sulfate proteoglycan (HSPG) and bikunin mRNA transcripts in renal cortex and medulla of control and nephrolithic rats using RT-PCR. PCR products were run individually on 1.5% agarose gels. Amplified HSPG, bikunin and cyclophilin cDNA from control group and nephrolithic group. NC, negative control. Liver cDNA served as positive control.

TABLE 2.
Relative expression levels of HSPG and Bikunin mRNA in rat kidney

HSPG				
	Cortex (1wk)	Medulla (1wk)	Cortex (2wks)	Medulla (2wks)
Control	82.23±2.92	975.49±61.90	1524.39±52.53	9198.02±625.84
Stone Group	351.34±24.38	1640.65±71.53	7443.99±867.51*	65941.00±2512.16**

Bikunin				
	Cortex (1wk)	Medulla (1wk)	Cortex (2wks)	Medulla (2wks)
Control	4.88×10 ⁵ ±2.25×10 ⁴	9.64×10 ⁵ ±4.33×10 ⁴	7.06×10 ⁵ ±2.83×10 ⁴	37.00×10 ⁵ ±4.16×10 ⁴
Stone Group	6.99×10 ⁵ ±4.38×10 ⁴	8.17×10 ⁵ ±3.13×10 ⁴	30.72×10 ⁵ ±1.10×10 ⁴ *	59.26×10 ⁵ ±9.05×10 ⁴ *

Results are given as mean ± S.E. (Transcripts number/μg of cDNA)
* $p < 0.05$, ** $p < 0.01$ (Control vs Stone group)

Fig. 2. Synthesis of HSPG mRNA transcripts is increased in rat kidneys during development of nephrolithiasis. (Left panel) QC-PCR of HSPG mRNA transcripts present in kidney tissues of control and nephrolithic rats after 2 wks (Right panel). Levels of transcripts increased about 22-fold in medulla of nephrolithic kidneys after 2 wks compared with control rats.

Fig. 3. Synthesis of bikunin mRNA transcripts increases in rat kidneys during nephrolithiasis. QC-PCR revealed increased expression of bikunin mRNA in medulla part of control and nephrolithic rat kidneys after 2 wks (Left Panel). Levels of transcripts increased about 2.4-fold after 2 wks under nephrolithic conditions compared with control rats (Right panel).

Fig. 4. Comparison of HSPG and bikunin gene expression between cortex and medulla in each kidney. A: relative expression of HSPG mRNA in the kidneys. Expression of HSPG in medulla is significantly higher than that of cortex in all groups. B: relative expression level of bikunin mRNA in medulla is significantly higher than in cortex in at 2 wks.

diluted by over 10-fold before HSPG cDNA could be amplified from an equal amount of nephrolithic kidney (right panel). These data indicate that approximately 1×10^5 HSPG mRNA transcripts/ μg cDNA were present in the nephrolithic rat renal medulla, while only about 1×10^3 HSPG mRNA transcripts/ μg cDNA were present in the normal rat renal medulla. Statistical analysis of the data revealed that the increased expression of HSPG mRNA transcripts in the kidney of cortex and medulla after the two-week treatment period was highly significant (cortex; 1500

vs. 7400 transcripts/ μg of cDNA, $p < 0.05$, medulla; 66000 vs. 9200 transcripts/ μg of cDNA, $p < 0.01$). Figure 3 shows that bikunin cDNA was amplified from normal rat renal medulla (2 wks) in the presence of QC-template DNA diluted more than 1×10^6 . In contrast, the QC-DNA template had to be diluted over 10-fold before bikunin cDNA could be amplified from an equal amount of nephrolithic rat renal medulla (2 wks). Statistical analysis of the data revealed that the increase in the expression of bikunin mRNA transcripts in the both the cortex and

medulla of the kidney after 2 wks of treatment was highly significant (cortex; 30×10^5 vs. 7×10^5 transcripts/ μ g of cDNA, $p < 0.01$, medulla; 59×10^5 vs.

37×10^5 transcripts/ μ g of cDNA, $p < 0.05$) (Table 2).

On the other hand, control and nephrolithic groups did not significantly differ at 1 wk. This sug-

Fig. 5. Polarized light photomicrographs of hematoxylin-eosin-stained medulla of rat kidneys. A, normal rat kidney. B, kidney after 2 wks of treatment showing calcium oxalate (CaOx) crystals in tubules (white arrow). Some tubules are dilated (arrow head). Photomicrographs of kidneys after immunohistochemical staining using anti-heparan sulfate monoclonal antibody (HepSS-1; C and D). Distal tubular lumens and collecting ducts of nephrolithic kidney are intensely stained by HepSS-1. Photomicrographs of kidneys after immunohistochemical staining using anti-ITI antibody (E and F). Staining was more intense than in normal rats. Distal tubules and collecting ducts are intensely stained.

gests that the level of HSPG and bikunin gene expression closely correlates with tubular epithelial damage.

Figure 4 compares HSPG and bikunin gene expression between the cortex and medulla in individual kidneys. Significantly more HSPG was expressed in the medulla than in the cortex of all groups. On the other hand, the relative expression level of bikunin mRNA in the medulla was significantly higher than in the cortex in the two-week groups.

Histochemical study

Figure 5 shows photographs of control and experimental rat kidneys after two weeks of treatment. Tubular morphology was normal in the kidneys of control rats and crystal formation was absent (Fig. 5A). Two weeks of treatment resulted in deposition of CaOx microliths in the renal tubules, which was associated with tubule dilation and damaged epithelial cells (Fig. 5B). We used anti-HS monoclonal antibody (HepSS-1) to immunohistochemically stain kidney tissues and localize HS under normal and nephrolithic conditions. Under normal conditions, renal tubules and Bowman's capsule were lightly and diffusely stained. The glomerular basement membrane (GBM) was not stained. In the nephrolithic rat kidneys, however, staining was more intense and localized to dilated tubules or those containing crystals (Figs 5C and D). Anti-I α I antibody was also used to immunohistochemically localize I α I under normal and nephrolithic conditions. Under normal conditions, cytoplasm of the distal tubules was lightly stained whereas proximal tubules and collecting ducts were not stained. However, staining intensity of the nephrolithic rat kidney was more intense than that of the normal kidney cortex and medulla (Figs 5E and F).

DISCUSSION

HS is present in tissues as a proteoglycan and in the urine as a metabolic turnover product of tissue proteoglycan. In the kidney, HS is the major acidic GAG constituent of the glomerular and tubular basement membrane (GBM and TBM), respectively [25]. HSPG in GBM, also known as perlecan, is important for maintaining the integrity of glomerular size and the charge barrier [26,27]. On the other hand, the localization and function of other types of HSPG, such as syndecan [28], remain unclear. Pyke et al. [29] investigated the expression of various proteo-

glycans in the normal rat kidney by in situ hybridization. They found that syndecan-4 is expressed in normal glomeruli, whereas syndecan-1 and perlecan mRNA were detected in the papillary area. Syndecan is localized on the surface of a variety of mammalian epithelial cells, where it is involved in maintaining epithelial morphology and stabilization [28].

The present study investigated expression levels of HSPG mRNA and its localization in nephrolithic rat kidney using QC-PCR and immunohistochemistry. The results showed that the level of HSPG mRNA expression apparently increased in the nephrolithic rat kidney. Moreover, HSPG expression was significantly higher in the medulla, than in the cortex in the nephrolithic rat kidney. Immunohistochemical studies revealed that production of HS was increased in both distal and proximal tubules during CaOx nephrolithiasis. On the other hand, bikunin, the light chain of inter- α -inhibitor, was initially isolated from normal human serum as a Kunitz-type protease inhibitor [30-33]. Free bikunin is present in both the plasma and urine of humans and rodents. Bikunin was originally identified as uronic acid protein (UAP), on the basis of its high uronic acid content [11-13]. Terminal amino acid sequence analysis of both rat and human UAP has revealed homology with bikunin [13]. Atmani et al. [11-13] reported that urinary bikunin could inhibit CaOx crystallization. Moreover, Iida et al. reported that expression of bikunin in the rat kidney is increased during CaOx nephrolithiasis [17]. The present study showed that the expression of HSPG and bikunin mRNAs is increased in both the cortex and medulla of the nephrolithic rat kidney after two weeks. Histochemical studies revealed that CaOx microlith formation and epithelial cell damage tended to increase in the medulla after two weeks. Moreover, immunohistochemical studies also revealed increased production of HS and bikunin/I α I-related proteins in both the distal and proximal tubules during nephrolithiasis. These data suggest that the increased expression of HSPG and bikunin play a significant role during CaOx stone formation. We postulate that the following mechanism is involved in HSPG mRNA expression during CaOx stone formation. CaOx crystals formed in the tubular lumen injure the epithelial layer containing HSPG and might induce increased HSPG mRNA expression to produce more HSPG to repair damaged urothelium. In fact, Stein and Parsons [34] reported that syndecan mRNA is expressed in bladder biopsy tissue from patients with interstitial cystitis (IC). IC

may thus reflect the inability of the bladder to repair its protective surface coating (GAGs and proteoglycans). They concluded that a defect in these protective substances on the urothelial surface is implicated in the expression of syndecan. More recently, Barsotti et al. [35] reported that the prevalence of glomerular sclerosis, mesangial proliferation and tubulointerstitial damage in kidneys of HS-treated rats is lower than controls. They proposed that HS acts as a renoprotective substance through its anti-proliferative and anti-inflammatory effects. Although the exact mechanism of HSPG gene expression remains unclear, our data support their proposal. On the other hand, the following mechanism may be involved in bikunin mRNA expression during CaOx stone formation. We reported that the bikunin gene is upregulated in renal epithelial cells exposed to oxalate. However, this phenomenon could not be shown in the cells exposed to calcium oxalate monohydrate (COM) crystals. These observations suggested that bikunin gene expression is stimulated by the presence of a high concentration of oxalate. In general, CaOx crystals form in the medulla rather than in the cortex. This correlates with the concentration of urinary salt. In the present study, the gene expression levels of bikunin in the medulla and cortex were similar. These observations support the notion that bikunin gene expression in the nephrolithic rat kidney is stimulated by hyperoxalic conditions. Although the exact mechanism of bikunin gene expression and production are uncertain, Marengo et al. [36] have reported that the urinary excretion of $I\alpha I$, as well as of heavy chains H1 and H2 is increased in men with CaOx stone disease. These findings indicate that the kidney responds to the stimulus provided by hyperoxaluria and CaOx crystallization by producing a variety of proteins including bikunin and other $I\alpha I$ -related proteins. Further studies are underway to elucidate the mechanisms whereby hyperoxaluria and CaOx crystal deposition stimulate the production of HS/HSPG and bikunin proteins in the kidneys.

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