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Role of inter-α-inhibitor and its related proteins in experimentally induced calcium oxalate urolithiasis. Localization of proteins and expression of bikunin gene in the rat kidney

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Summary Our earlier studies indicated that members of the inter-α-inhibitor (IαI) family of glycoproteins may play an important role in urolithiasis. Indeed bikunin, the light chain of IαI is a potent inhibitor of calcium oxalate crystallization. In order to understand this role, the distribution of IαI and its related proteins, as well as the expression of bikunin, were studied in normal and nephrolithic rats. In normal rats, IαI immunoreactivity was located mainly in proximal tubules. However, in nephrolithic rats, in addition to proximal tubules, the staining was intensively extended to tubules in the corticomedullary junction. Furthermore, by using polymerase chain reaction technique, we demonstrated that gene encoding for bikunin was activated in kidneys of nephrolithic rats. We have previously demonstrated increased staining for osteopontin in association with calcium oxalate crystal deposition in rat kidneys. Others have shown an increase in osteopontin production by renal epithelial cells on exposure to calcium oxalate crystals. Based on these observations we conclude that kidney cells possess an auto-defense system against calcium oxalate crystallization and stone formation in which members of the IαI family may be closely involved.

Key words Nephrolithiasis · Kidneys · Calcium oxalate crystallization · Inter-α-inhibitor · Bikunin

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Introduction

Urinary stone formation or urolithiasis is a common human urological disorder that is still characterized by conflicting experimental and clinical findings. Pathogenesis of urolithiasis involves nucleation, growth, aggregation, and retention of crystals within the urinary tract. Several macromolecular modulators such as glycoproteins control these processes and promote or inhibit them [1, 7, 12, 17, 22, 29]. Among these modulators is bikunin, a glycoprotein that inhibits calcium oxalate (CaOx) crystallization [2–5]. Bikunin belongs to the inter-α-inhibitor (IαI) family, a serine protease inhibitor present in plasma. The IαI family has five major well-characterized members [27]. Three members of the family contain bikunin covalently linked by a chondroitin-4-sulfate glycan bond to one or two heavy or H chains (Fig. 1). The fourth member of H4 polypeptide is found free. Bikunin is also found in the free state, in plasma as well as urine. At least five genes namely α1-microglobulin/bikunin precursor gene (AMBP), H1, H2, H3, and H4, are involved in the synthesis of various members of the IαI family [27]. The genes encoding for H1, H3, and H4 are on chromosome 3. H2 gene is on chromosome 10 and bikunin gene is on chromosome 9. The AMBP mRNA encodes a precursor for α1-microglobulin (α1m) and bikunin. The precursor undergoes a cleavage that releases the two proteins.

Inter-α-inhibitor (IαIH1,2,B), with a molecular weight of about 220 kDa, contains H1, H2, and bikunin [10, 25, 28]. Another macromolecule related to the IαI family has also been found and named pre-α-inhibitor (IαIH3, B) [14]. It has a molecular weight of 125 kDa and is made of a heavy chain H3 and bikunin. Members of the IαI family are synthesized mainly in the liver and excreted in plasma. This finding was confirmed by molecular biology techniques [26]. However, in addition to liver, the gene encoding for H3 is also expressed in the brain [25]. Furthermore, mRNA encoding for the pre-

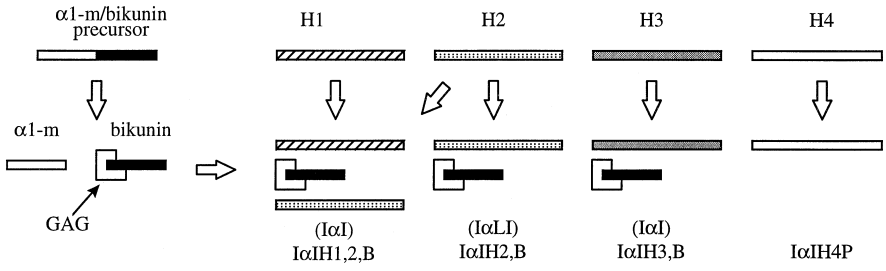

Fig. 1 Current view of the structure of IαI family and its related derivatives according to Salier et al. [27]. α1-M α1-microglobulin, *H* heavy chain, *I*αI inter-α-inhibitor, *P*αI pre-α-inhibitor. *P* Precursor, *GAG* Glycosaminoglycon chain

cursor α1-microglobulin/bikunin was found in rats at a high level in the liver and kidney, and at a low level in the brain and testis [20]. Also, the expression of this precursor was found at a high level in the liver and a low level in the pig's stomach [30]. Under normal circumstances by using immunohistochemical techniques, IαI-related proteins were found in different organs including kidneys in which the immunoreactivity was observed especially in renal proximal tubules [8, 23, 32].

The aim of the present study was (1) to compare the immunohistological distribution of IαI related proteins in the kidney of normal and nephrolithic rats, and (2) to study the expression of the bikunin gene in the kidney of nephrolithic rats.

Materials and methods

Induction of hyperoxaluria and CaOx crystals deposition in rat kidneys

Eleven male Sprague Dawley rats weighing 120–125 g were housed in metabolic cages and allowed to acclimatize to their environment for 3 days prior to the start of the experiment. During the first week, animals were given regular drinking water ad libitum. On the day 8, animals were given 0.75% ethylene glycol in the drinking water to induce hyperoxaluria and CaOx crystals deposit. The experiment was conducted for 6 weeks. During the experimental period, animals were provided ground regular rat chow. A daily weight, urine collection on ice, pH, and light microscope examination were done. Urinary constituents were determined in the end of each week by ion chromatography and atomic absorption. All rats were killed by intraperitoneal injections of sodium pentobarbital. Kidneys were perfused with saline solution and removed. One kidney was sectioned into cortex, medulla, and papilla, frozen in liquid nitrogen, and stored at −70°C. The other kidney was fixed in either 10% formalin or half-strength Karnovsky's fixative solution. They were examined for crystal deposit and localization of IαI related proteins on 5 μm thick sections.

Immunohistochemical staining

Tissue sections were deparaffinized in two changes of xylene an rehydrated through a graded series of ethanol. After three washes in PBS of 5 min each, endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide during 30 min at room temperature. Slides were again washed three times in PBS and treated with 0.1% trypsin (Sigma Chemicals, Mo.) for 10 min at 37°C. Nonspecific binding sites were blocked by incubation with 2% bovine serum albumin in PBS containing 15 μl/ml nonimmune goat serum for 1 hour and then incubated with anti-IαI antibodies (Accurate Chemical and Scientific, N.Y.) at a dilution of 1:100 in the same buffer without goat serum for 1 hour at room temperature. After rinses with PBS sections were incubated with goat anti-HRPO conjugate (Fisher Scientific, Pa.) at a dilution of 1:200 for 30 min. Color was developed by using 3,3′-diaminobenzidine (Sigma Chemicals, Mo.) as the substrate for 1 min and then rinsed in PBS. Sections were counterstained with hematoxylin, dehydrated through progressive alcohol washes, and mounted with Permount (Fisher Scientific, Pa.).

Polymerase chain reaction (PCR) amplification of bikunin mRNA sequence

Total mRNA were isolated from frozen, pulverized rat organs using a Boehringer Mannheim kit. Approximately 2 μg of total mRNA was used as template for cDNA synthesis by reverse transcription. Specific oligonucleotide primers were designed according to the published sequence of bikunin mouse cDNA [9] which are as follow: 3′-GCAGTGCTGCCCCAAGAG-5′ and 5′-TGACGCATAGTCAAGGAGCAT-3′. PCR reactions were conducted for 35 cycles. The PCR products were subjected to electrophoresis on 1% agarose gel containing ethidium bromide to visualize the bands.

Results and discussion

IαI is the most studied and well-characterized serum proteinase inhibitor. It is a large serine proteinase inhibitor which is believed to be the precursor of smaller inhibitors present in different biological fluids [15]. These

Table 1 Effect of ethylene glycol on urinary chemistries determined by ion chromatography. ** Significant $P < 0.05$, ** $P < 0.005$ *** very significant $P < 0.0005$. Values were compared between week 1 vs. week 2 and week 1 vs. week 6

Urinary constituents (mg/24 h)	Week 1	Week 2	Week 6
Sodium	65.22 ± 1.95	61.04 ± 1.51	68.52 ± 2.35
Ammonia	9.90 ± 0.37	10.58 ± 0.43	12.29 ± 0.81**
Potassium	187.20 ± 4.69	181.01 ± 3.79	184.28 ± 16.27
Magnesium	7.52 ± 0.51	7.93 ± 0.91	8.43 ± 1.34
Calcium	4.63 ± 0.47	2.25 ± 0.22***	1.93 ± 0.26***
Sulfate	43.21 ± 1.28	45.45 ± 1.44	60.51 ± 1.93***
Oxalate	1.71 ± 0.05	8.01 ± 1.23***	9.82 ± 1.71***
Phosphate	15.43 ± 0.93	20.62 ± 1.50**	17.70 ± 1.21
Citrate	97.25 ± 3.71	68.56 ± 3.52***	38.12 ± 5.13***
pH	6.76 ± 0.04	6.50 ± 0.05***	6.33 ± 0.09***

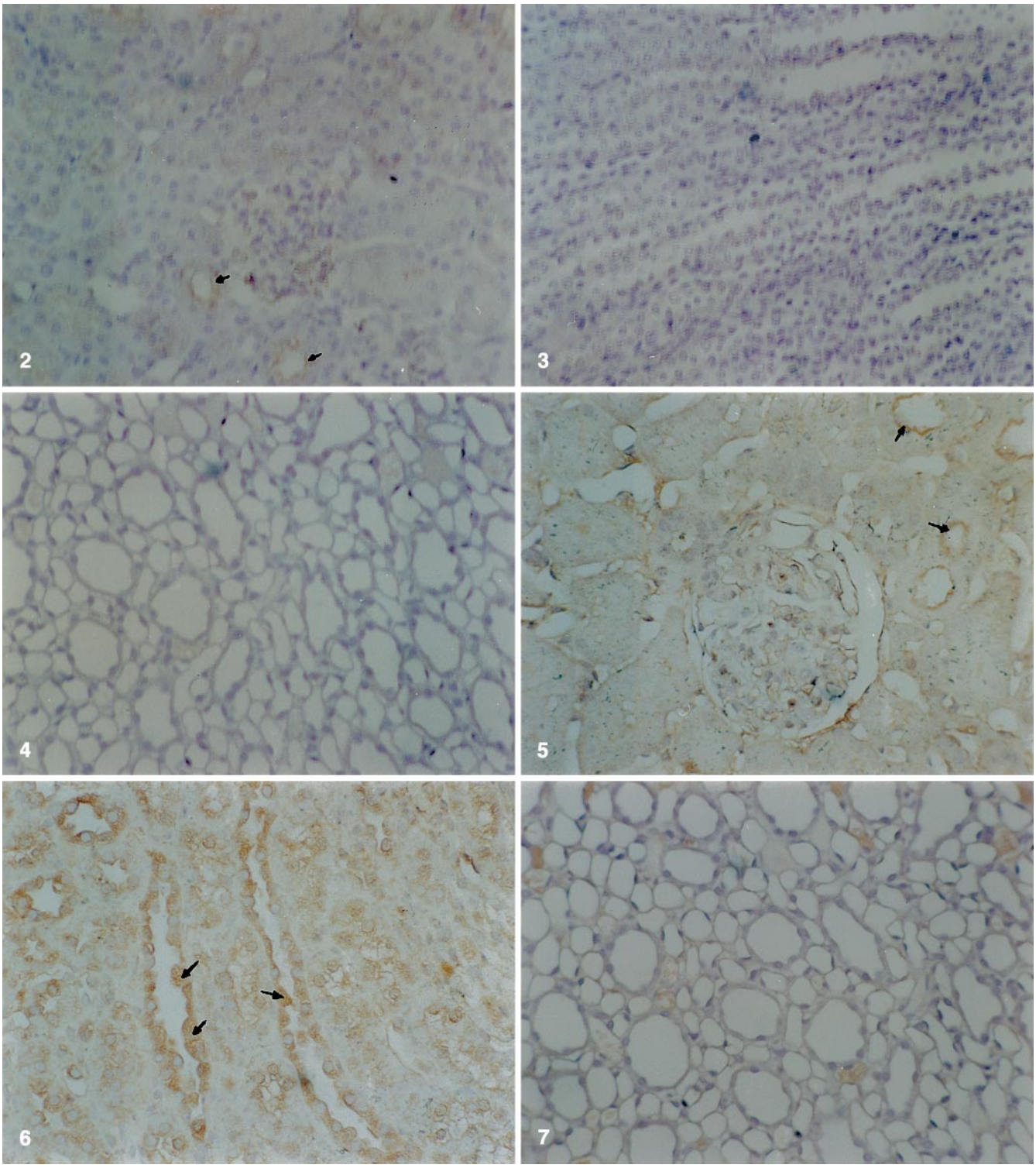

Fig. 2 Immunohistochemical localization of IαI-related proteins in the cortex of normal kidney rat. Weak staining is observed in proximal tubules (*arrows*)

Fig. 3 Immunohistochemical localization of IαI-related proteins in the corticomendullary junction of normal kidney rat. No staining is observed

Fig. 4 Immunohistochemical localization of IαI-related proteins in the papilla of normal kidney rat. No staining is observed

Fig. 5 Immunohistochemical localization of IαI-related proteins in the cortex of nephrolithic kidney rat. Intense staining is observed in proximal tubules (*arrows*)

Fig. 6 Immunohistochemical localization of IαI-related proteins in the corticomedullary junction of nephrolithic kidney rat. Intense staining is observed (*arrows*)

Fig. 7 Immunohistochemical localization of IαI-related proteins in the papilla of nephrolithic kidney rat. No staining is observed

inhibitors appear to be involved in the inhibition of various enzymes which are activated during diverse pathological circumstances such as cancer, fibrinolysis, and inflammation [11, 18, 24]. Distribution and localization of IαI-related proteins have been a focus of several investigations. Indeed, IαI immunoreactivity has been shown in liver, kidney, testis, gross intestine, cutis, and brain [8, 23, 32]. Its presence in various tissues suggests that it may fulfill different physiological functions. So far, however, many of these roles remain tentative. In the kidneys, IαI immunoreactivity was found essentially in proximal tubules [8, 23, 32]. According to our earlier studies, bikunin and other members of the IαI family may be involved in urolithiasis [5]. We have shown that bikunin inhibits CaOx crystallization and bikunin and other IαI-related proteins are included in the matrix of CaOx crystals experimentally induced in human urine [6]. We postulate that the presence of IαI-related proteins in the kidney may be beneficial against crystallization in the renal tubules. In order to understand the role of IαI family in urolithiasis, experiments were carried out in normal and nephrolithic rats. The later were used to mimic stone formation in humans by experimental induction of hyperoxaluria, which is the main risk factor for nephrolithiasis. Our objective was to investigate the occurrence of any specific changes in the localization of IαI-related proteins and the expression of bikunin gene in nephrolithic rat kidneys as a consequence of hyperoxaluria and CaOx crystal deposition.

Ethylene glycol administration caused an increase in urinary oxalate excretion and CaOx supersaturation (Table 1) similar to our earlier observations [21]. The light microscopic analysis of urine specimens showed a high amount of calcium oxalate monohydrate and dihydrate crystals. The examination of kidneys after 6 weeks of ethylene glycol administration showed that all rats formed crystal deposits, which were located essentially in corticomedullary junction, papilla, papillary tip, and fornices.

Examination of normal kidneys of control rats showed the expected pattern of IαI immunoreactivity in the proximal tubules only (Fig. 2–4). Our finding is in agreement with studies performed by other investigators [8, 23, 32]. In the nephrolithic rats our major finding was that, in addition to the proximal tubules, IαI immunoreactivity was intensely localized in the renal tubular segments of the corticomendullary junction (Fig. 5–7). These results are similar to earlier reports from our laboratory [16] of increased osteopontin expression in kidneys of nephrolithic rats. Osteopontin is another macromolecule that modulates biomineralization. However, the increased localization does not necessarily mean production by the renal epithelial cells. In order to prove this point, total mRNA was extracted from different parts of the kidney. In parallel, total liver mRNA was also extracted as a control. PCR technique was used to amplify the bikunin gene. PCR products obtained by using either 3′ primer or both 3′ and 5′ primers were subjected to electrophoresis on agarose gel. As it can be seen in Fig. 8, an intense band at 450 bp corresponding to the bikunin gene appears for both liver and kidney of nephrolithic rats. This suggests that the bikunin gene is upregulated in response to hyperoxaluria and CaOx crystals deposition. Our finding is in conflict with results obtained by Salier et al. [26] who demonstrated that IαI and its related proteins are synthesized only in the liver. This discrepancy may be explained by the fact that Salier and colleagues studied the expression of these proteins 'in normal rat kidneys. Our laboratory has also shown an increase in bikunin gene expression in LLC-PK_1 and MDCK cells exposed to oxalate and CaOx crystals [19]. These two cell types were used as representative of epithelia lining the proximal tubule and collecting ducts, respectively. Based on our results described here and elsewhere we hypothesize that the bikunin gene is upregulated by hyperoxaluria and calcium oxalate crystal deposition.

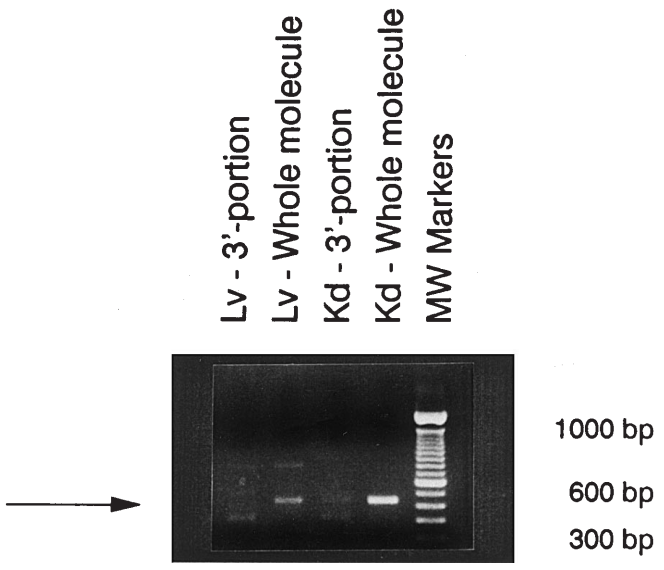

Fig. 8 Polymerase chain reaction products were subjected to electrophoresis on agarose gel. *Arrow* indicates the position of the bikunin gene. *MW* molecular weight markers

A number of studies are currently underway in our laboratory to determine increased production of bikunin and localize the site of its production. It is, however, apparent that members of the IαI family are involved in urolithiasis. The synthesis of bikunin protein appears to be stimulated following the activation of its gene by hyperoxaluria and/or CaOx crystal deposition. A similar increase has been seen in osteopontin expression by nephrolithic rats. The kidneys appear to respond to biomineralization challenge by producing macromolecular modulators including members of the IαI family of proteins.

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References

1. Atmani F, Lacour B, Drüeke T, Daudon M (1993) Isolation and purification of a new glycoprotein from human urine inhibiting calcium oxalate crystallization. Urol Res 21:61
2. Atmani F, Lacour B, Strecker G, Parvy P, Drüeke T, Daudon M (1993) Molecular characteristics of uronic-acid-rich protein, a strong inhibitor of calcium oxalate crystallization in vitro. Biochem Biophys Res Comm 191:1158
3. Atmani F, Khan SR (1995) Characterization of uronic-acid-rich inhibitor of calcium oxalate crystallization isolated from rat urine. Urol Res 23.95
4. Atmani F, Mizon J, Khan SR (1996) Identification of uronic-acid-rich protein as urinary bikunin, the light chain of inter-α-inhibitor. Eur J Biochem 236:984
5. Atmani F, Mizon J, Khan SR (1996) Inter-α-inhibitor: a protein family involved in the inhibition of calcium oxalate crystallization. Scanning Microsc 10:425
6. Atmani F, Khan SR (1996) Inter-α-inhibitor, another serum protein with potential involvement in calcium oxalate nephrolithiasis. J Am Soc Nephrol 7:1798
7. Bowyer RC, Brockis JG, McCulloch RK (1979) Glycosaminoglycans as inhibitors of calcium oxalate crystal growth and aggregation. Clin Chim Acta. 95:23
8. Businaro R, Leali FMT, DeRenzis G, Pompili E, Pagliari G, Menghi G, Fumagalli L (1992) Inter-alpha-trypsin inhibitor-related immunoreactivity in human tissues and body fluids. Cell Mol Biol 38:436
9. Chan P, Salier JP (1993) Mouse α-1-microglobulin/bikunin precursor: cDNA analysis, gene evolution and physical assignment of the gene next to the orosomucoid locus. Biochim Biophys Acta 1174:195
10. Diarra-Mehrpour M, Bourguignon J, Sesboüé R, Matte MG, Passage E, Salier JP, Martin JP (1989) Human plasma Inter-α-trypsin inhibitor is encoded by four genes on three chromosomes. Eur J Biochem 179:147
11. Dietl T, Dobrinski W, Hochstrasser K (1979) Human Inter-α-trypsin inhibitor. Limited proteolysis by trypsin, plasmin, kallikrein and granulocytic elastase and inhibitory properties of the cleavage products. Hoppe-Seyler's Z Physiol Chem 360:1313
12. Doyle IR, Marshall VR, Dawson CJ, Ryall RL (1995) Calcium oxalate crystal matrix extract: the most potent macromolecular inhibitor of crystal growth and aggregation yet tested in human undiluted urine in vitro. Urol Res 23 human:53
13. Eleuteri AM, Angeletti M, Fioretti E (1994) Proteinase inhibitors of the Kunitz family in fallow deer organs: a comparative study. Comp Biochem Physiol 107B:539
14. Enghild JJ, Thagersen IB, Pizzo SV, Salvesen G (1989) Analysis of inter-α-trypsin inhibitor and a novel trypsin inhibitor, pre-α-trypsin inhibitor, from human plasma. Polypeptide chain stoichiometry and assembly by glycan. J Biol Chem 264:15975
15. Gebhard W, Hochstrasser K (1986) Inter-α-trypsin inhibitor and its close relatives. In: Proteinase inhibitors. Amsterdam, Elsevier, pp 389–401
16. Gokhale JA, Glenton PA, Khan SR (1996) Localization of Tamm-Horsfall protein and osteopontin in a rat nephrolithiasis model. Nephron 73:456
17. Hess B (1994) Tamm-Horsfall glycoprotein and calcium nephrolithiasis. Miner Electrolyte Metab 20:393
18. Hochtrasser K, Bretzel G, Feuth H, Hilla W, Lempart K (1976) The inter-α-trypsin inhibitor as precursor of the acid-stable proteinase inhibitors in human serum and urine. Hoppe-Seyler's Physical Chem 357:153
19. Iida S, Johnson-Tardieu J, Glenton PA, Byer K, Peck AB, Khan SR (1997) Molecular detection of bikunin in normal and oxalate exposed kidneys and renal epithelial cells. J Am Soc Nephrol 8:563
20. Kastern W, Björck L, Åkerström B (1986) Developmental and tissue-specific expression of α1-microglobulin mRNA in the rat. J Biol Chem 261:15070
21. Khan SR, Hackett RL (1987) Urolithiasis of mixed foreign body stones. J Urol 138:1321
22. Nakagawa Y, Abram V, Kezdy FJ, Kaiser ET, Coe FL (1983) Purification and characterization of the principal inhibitor of calcium oxalate monohydrate crystal growth in human urine. J Biol Chem 258:12594
23. Ødum L (1989) Immunohistochemical investigation of inter-α-trypsin inhibitor in urinary tract. APMIS 97:375
24. Potempa J, Kwon K, Chawia R, Travis J (1989) Inter-α-trypsin inhibitor. Inhibition spectrum of native and derived forms. J Biol Chem 264:15109
25. Salier JP, Diarra-Mehrpour M, Sesboüé R, Bourguignon J, Benarous R, Ohkubo I, Kurachi S, Kurachi K, Martin JP (1987) Isolation and characterization of cDNAs encoding the heavy chain of human inter-α-trypsin inhibitor (IαTI): unambiguous evidence for multipolypeptide chain structure of IαTI. Proc Natl Acad Sci USA 84:8272
26. Salier JP, Chan P, Raguenez G, Zwingman T, Erickson RP (1993) Developmentally regulated transcription of the four liver-specific genes for inter-α-trypsin inhibitor family in mouse. Biochem J 296:85
27. Salier JP, Rouet P, Raguenez G, Davesu M (1996) The inter-α-inhibitor family: from structure to regulation. Biochem J 315:1
28. Schreitmüller T, Hochstrasser K, Reisinger PWM, Wachter E, Gebhard W (1987) cDNA cloning of human inter-α-trypsin inhibitor discloses three different proteins. Biol Chem Hoppe-Seyler 368:963
29. Shiraga H, Min W, Vandusen WJ, Clayman MD, Miner D, Terrell CH, Sherbotie JR, Foreman JW, Przysiecki C, Neilson EG, Hoyer JR (1992) Inhibition of calcium oxalate crystal growth in vitro by uropontin: another member of the aspartic acid-rich protein superfamily. Proc Natl Acad Sci. USA 89:426
30. Tavakkol A (1991) Molecular cloning of porcine α1-micoglobulin/HI-30 reveals developmental and tissue-specific expression of two variant messenger ribonucleic acids. Biochim Biophys Acta 1088:47
31. Thamilselvan S, Hackett RL, Khan SR (1997) Lipid peroxidation in ethylene glycol induced hyperoxaluria and calcium oxalate nephrolithiasis. J Urol 157:1059
32. Yoshida E, Sumi H, Tsushima H, Maruyama M, Mihara H (1991) Distribution and localization of inter-α-trypsin inhibitor and its active component acids-stable proteinase inhibitor: comparative immunohistochemical study. Inflammation 15:71