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**Yasushi Nakagawa · Mauricio Carvalho · Prida Malasit
Sumalee Nimmannit · Suchai Sritippaywan
Somkiat Vasuvattakul · Somchai Chutipongtanate
Vipada Chaowagul · Sanga Nilwarangkur**

Kidney stone inhibitors in patients with renal stones and endemic renal tubular acidosis in northeast Thailand

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Abstract Distal renal tubular acidosis (dRTA) is generally associated with hypercalciuria, hypocitraturia, and nephrolithiasis. Our intention was to study glycosaminoglycans (GAGS) and nephrocalcin (NC), two well-known crystal growth inhibitors, in a population with endemic dRTA and nephrolithiasis in northeast (NE) Thailand. We studied 13 patients, six with dRTA and seven with nephrolithiasis with normal or undefined acidification function. Six healthy adults living in the same area as the patients and another six from the Bangkok (BKK) area were used as controls. We measured urinary pH, ammonia, calcium, citrate, magnesium, oxalate, potassium, sodium and uric acid. GAGS were determined by an Alcian blue precipitation method and were qualitated by agarose gel electrophoresis after being isolated using 5% cetyltrimethylammonium bromide at pH 6.0. NC isoforms were isolated as previously described by Nakagawa et al. Citrate was higher in BKK controls ($p < 0.04$). There was a striking difference among GAGS from BKK when compared with other groups (103.85 ± 10.70 vs. 23.52 ± 8.11 for dRTA, 22.36 ± 14.98 for kidney stone patients and 14.73 ± 2.87 mg/ml in controls from the NE region, ($p < 0.0001$). dRTA and stone-forming patients excrete proportionally more (C + D) than (A + B) NC isoforms ($p < 0.05$). Also, their NC showed a 100-fold weaker binding capacity of calcium oxalate monohydrate crystals. The ratio of chondroitin sulfate/heparin sulfate in GAGS was approximately 9/1. In addition to the traditional risk factors for nephrolithiasis in dRTA, GAGS and NC might play an important role in the pathogenesis of stone formation in this population.

Keywords Renal tubular acidosis · Kidney stone · Kidney stone inhibitors · Nephrocalcin isoforms

Y. Nakagawa (✉) · M. Carvalho
Kidney Stone Program, MC 5100,
Division of Biological Sciences and the Pritzker School of Medicine, University of Chicago,
5841 S. Maryland Ave., Chicago, IL 60637, USA
E-mail: ynakagaw@medicine.bsd.uchicago.edu
Fax: +1-773-7025818

P. Malasit · S. Nimmannit · S. Sritippaywan
S. Vasuvattakul · S. Chutipongtanate
Renal Unit, Department of Medicine,
Medical Molecular Biology Unit,
Office for Research & Development, Faculty of Medicine,
Siriraj Hospital, Mahidol University, Bangkok, Thailand

V. Chaowagul · S. Nilwarangkur
Sanpasitthiprasong Hospital,
Ubolrajathani, Thailand

Introduction

Distal renal tubular acidosis (dRTA) is a condition characterized by an abnormality in the generation and maintenance of a hydrogen ion gradient by the distal tubule which causes low urine ammonium excretion [1]. It is a heterogeneous disorder that may be hereditary, idiopathic or secondary to a variety of conditions [1]. Nephrolithiasis and nephrocalcinosis are common complications associated with dRTA, but the pathogenic mechanism is unknown [2, 3]. Alkaline urine, hypercalciuria and hypocitraturia are reported to be risk factors associated with renal stone formation [2], but the findings are not universal in every dRTA patient [4].

We have previously reported a high prevalence (2.2∼3.4%) of endemic dRTA in a population living in the northeast (NE) of Thailand [5]. Hypocitraturia and kidney stones were found in 30 and 15% of the population, respectively. No evidence of hypercalciuria or hyperoxaluria was found in these renal stone patients [6, 7]. We proposed that potassium deficiency, which was found in the area, might contribute to the pathogenesis of endemic dRTA [8].

Little is known about the role of macromolecular inhibitors in stone-forming patients with dRTA. A recent study demonstrated significant reduction of

uromucoid and glycosaminoglycans (GAGS) in this group of patients [9]. We investigated further whether nephrocalcin and GAGS, which are both potent renal stone inhibitors, also contribute to the pathogenesis of nephrolithiasis commonly found in this area.

Patients and methods

Patients and diagnosis criterion

The study comprised of 13 patients, six with dRTA with or without nephrocalcinosis (five females and one male, age 29–58 years, mean 45.17 ± 11.92 years) and seven with nephrolithiasis with normal or undefined acidification function (two females andfive5 males, age 26–62 years, mean 42.71 ± 13.46 years). All were indigenous inhabitants within Ubolrajathani, a major province in the northeast of Thailand. Considering that geographic, social background and genetic homogeneity in this population may have generated peculiar lifestyle and eating habits, we used two healthy control groups: one consisted of six villagers living in the same province and another group consisted of six individuals living in Bangkok (BKK). dRTA was defined by a low rate of urinary ammonium excretion and inability to lower the urine pH below 5.5 in the presence of systemic acidosis (serum bicarbonate below 20 mEq/L) or after an acute acid loading test as described by Wrong and Davies [10]. All subjects were on their usual diets and all medications were stopped at least 2 weeks prior to the study.

Urine chemistry

Freshly voided urine samples were collected and preserved under thymol. The urinary pH was measured by a combination electrode attached to a Beckman pH meter (model ϕ71). Ammonia, calcium, chloride, creatinine, magnesium, phosphate, potassium, sodium and uric acid were determined using a Beckman Synchron X5 (Beckman-Coulter Instruments, Brea, CA, USA). Oxalate (oxalate oxidase, Sigma Diagnostics, St. Louis, MO, USA) and citrate (citrate lyase, Boehringer Mannheim, Indianapolis, IN, USA) were measured by enzymatic methods modified for automation, adapted to the Beckman CX5.

Quantitative determination of glycosaminoglycan (GAGS)

Urinary GAGS was determined by an Alcian blue method described by Whiteman [11]. An aliquot of 100 µl of urine sample was mixed with 1 ml of aqueous solution containing 0.05% Alcian blue (w/v) and 50 mM $MgCl_2$, pH 5.8, and kept overnight at room temperature. The mixture was centrifuged at 3,000 rpm for 15 min using a Beckman table top centrifuge (Spinchron centrifuge). The collected Alcian blue-GAGS complex was washed three times with 1 ml of absolute ethanol, and finally the precipitate was dissolved in 1 ml of 7.5% aqueous SDS solution. The optical density was measured at 620 nm using a Beckman spectrophotometer (DU 640). Calibration curve was prepared using 10–100 µg of chondroitin sulfate as a standard.

Qualitative analysis of GAGS by agarose gel electrophoresis

GAGS were precipitated from urine using 5% cetyltrimethylammonium bromide at pH 6.0, 4°C overnight and subjected to agarose gel (0.5% on 7.5×8.5 cm plate) electrophoresis as described by Maccari et al. [12]. The Alcian blue stained bands were cut out and dissolved in 1 ml of 7.5% aqueous SDS solution, and optical density was measured at 620 nm using Beckman spectrophotometer.

Isolation of NC isoforms

Each urine was subjected to anion exchange (DEAE cellulose) chromatography and two steps of molecular sieve column chromatography for isolation and purification of four NC isoforms as reported previously [13]. Salt gradient was monitored by using a conductivity meter (Radiometer Model CDN210). Protein elution was monitored by absorption at 280 nm or 230 nm, and calcium oxalate crystal growth inhibition was measured by using ^{14}C-oxalate incorporation method [13]. Isolated NC concentration was determined by alkaline hydrolysis followed by ninhydrin color reaction using 5–20 µg of BSA as standards [13]. Phosphate content was determined by the method described by Ames [14]. The color was developed by using Fiske-Subbarow reagent and 0.01 M KH_2PO_4 was used for preparing calibration standard ranging from 50 to 500 µmole of phosphate concentration.

Dissociation constant of NC isoforms toward COM crystals

The initial velocity of the COM crystal growth inhibitory activity was determined by a spectrophotometric method for determining a second order reaction rate [13]. Dissociation constant of each NC isoform was calculated from Langmuir isotherm type plot by using the initial velocity vs. inhibitor concentration.

Surface tension measurement by Lauda film balance

Surface pressure at the air-water interface was measured by using Lauda film balance (Brinkman Instruments, Westbury, NY, USA). NC isoform, 100 µg, was spread over the surface of 327 cm^2 of 0.01 M Tris-HCl, pH 7.3, containing 0.1 M NaCl. The surface pressure was measured by compressing a protein monolayer at the rate of 2.2 cm^2/s at 27°C. The pressure changes were monitored and recorded by computer. Dissociation constant and collapsing pressure were measured in some subjects of each group.

Statistical analysis

Clinical results are given as mean ± standard error of mean (SEM). For comparisons among group means, analysis of variance (ANOVA) was employed.

Results

Urine chemistry

Table 1 summarizes the pH and the concentrations of urinary chemical compositions. Ammonia and potassium excretion by the dRTA group were significantly lower when compared with the controls. Urinary citrate and GAGS were higher in the controls from BKK when compared with patient groups ($p < 0.04$ and $p < 0.0001$, respectively). GAGS concentration of this group was almost five times higher compared with those in the dRTA group, nephrolithiasis and controls in NE group. The other values were not significantly different among the groups studied. GAGS of all three groups showed ~90% of chondroitin sulfate and ~10% of heparan sulfate. Chondroitin sulfate migration distances of dRTA patients were more variable compared with controls. This indicates that the sulfation degree might be different.

Table 1 Chemical analyses of urine specimens

	dRTA	Stone former	NE control	BKK control	*P* values
N	6	7	6	6	-
PH	6.27 ± 0.87	6.24 ± 0.35	6.19 ± 0.19	6.33 ± 0.52	NS
Ca (mg/dL)	4.93 ± 3.23	6.34 ± 2.70	4.92 ± 4.20	5.37 ± 2.56	-
Ox (mg/dL)	0.39 ± 0.24	0.46 ± 0.15	0.35 ± 0.20	0.57 ± 0.38	-
Mg (md/dL)	2.28 ± 0.80	3.06 ± 1.40	2.23 ± 1.62	3.76 ± 1.96	-
Cit (mg/dL)	1.60 ± 0.87	2.11 ± 1.60	2.13 ± 1.62	17.07 ± 10.18	< 0.04
K (mmol/L)	6.92 ± 1.74	17.01 ± 10.56	11.96 ± 6.45	19.02 ± 8.90	< 0.001
Amm (mmol/L)	3.25 ± 0.93	10.84 ± 8.95	11.39 ± 4.74	9.75 ± 3.68	< 0.01
GAGS (mg/ml)	2.00 ± 9.82	22.36 ± 14.98	14.73 ± 2.87	103.85 ± 10.70	< 0.0001

Separation of NC isoforms

Nephrocalcin isoforms were separated from samples by DEAE cellulose column chromatography using a linear NaCl gradient. Four isoforms of NC A—D were separated as we reported previously [13, 15]. dRTA and stone-forming patients proportionally excreted more (C + D, ~67%) isoforms than (A + B, ~33%) isoforms ($p < 0.05$). Control NE group excreted isoforms (A + B, 47.3%) and (C + D, 52.7%) at almost equal ratios, and the BKK control group showed slightly higher (A + B, 54.1%) than (C + D, 45.9%) (Fig. 1). Each isoform was further purified using two steps of molecular sieve column chromatographys [13]. The major peaks were eluted at mol. Wt. of ~14 KD.

Dissociation constant of NC isoforms toward calcium oxalate monohydrate

Dissociation constants of control isoforms were in the order of 10^{-7} M, but those of stone formers were 10^{-6} M. However, the dissociation constant in dRTA patient was 10^{-5} M, which represents a 100-fold weaker binding capacity to calcium oxalate monohydrate crystals.

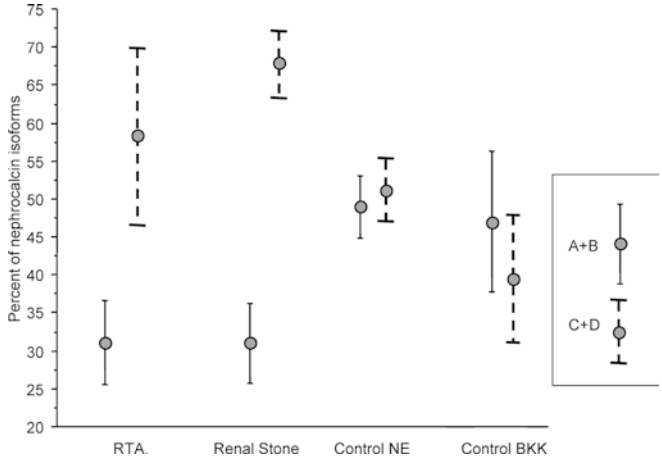

Fig. 1 Mean and standard error bars of the percentage of each group of NC isofroms (A + B) and (C + D) in the four groups of patients and controls. NC isoforms were separated by DEAE cellulose column using a linear NaCl gradient from 0.1 to 0.4 M in 0.02 M Tris-HCl buffer, pH 7.3. The calcium oxalate crystal growth inhibitory activity was measured by ^{14}C-oxalate incorporation into seed crystals. The inhibitory activity areas of four isoforms were integrated and expressed in % of each urine samples. Compare between (A + B) and (C + D) $p < 0.05$

Surface properties of NC isoforms

Isoform A of BKK controls built-up pressure early and collapsed at 59.7 mN/m, which indicates that the molecule has a strong amphiphilic property. During the compressing process, molecules arranged their position at the water-air interface as hydrophilic side in water and hydrophobic side over water, then the film collapsed. In the case of Isoform B, the molecule had less amphiphilic property compared with Isoform A, and the molecules dissolved into a water layer as pressure increased, and finally collapsed. Isoforms C and D are the same as Isoform B; the less amphiphilic nature causes more water solubility and less collapsing pressure. The tracing of surface tension curve of isoform A was superimposable after repeated compression and expansion. In contrast, those of isoforms B, C and D decreased after repeated compression and expansion. This indicates isoform A has strong amphiphilicity and forms a stable monolayer at the air-water interface, but the others are less amphiphilic and gradually dissolve into the water phase during the compression process. In general, Isoform A has little phosphorylation and Isoform D is highly phosphorylated, and collapsing pressure decreases as the degree of phosphorylation increases as reported previously [15, 16]. NC isoforms isolated from dRTA patients contained higher phosphate residues per mole of inhibitors and showed less amphiphilicity and higher dissociation constants toward CaOx compared with the BKK and NE controls.

Discussion

In previous studies, we described the high prevalence of dRTA with and without renal stone and/or nephrocalcinosis and also renal stone with normal acidification in northeastern Thailand [5]. In addition, we found no evidence of hypercalciuria and hyperoxaluria [17], similar to the study conducted by others in the same area [6, 7].

Urinary citrate has been identified as a potent inhibitor of calcium stone formation. Hypocitraturia affects 18–40% of patients with nephrolithiasis. Citrate binds to calcium, decreasing calcium oxalate and phosphate formation [18]. Urinary citrate is freely filtered in the glomerulus and reabsorbed in the proximal tubule. Metabolic acidosis or hypokalemia causes hypocitraturia with concomitant adaptive increases in proximal tubular Na-citrate cotransport activity [19]. Our finding of hypocitraturia in patients with dRTA, stone formers and normal villagers is very interesting because previous work has shown that a state of potassium deficiency exists in this population and might be one of the primary factors responsible for the hypocitraturia, which in turn increases the susceptibility to stone formation [5]. Mean serum potassium values of these dRTA patients were 3.60 ± 0.41 mmol /L, which were lower when compared with control values of 4.33 ± 0.36 mmol/L ($p < 0.05$).

Urinary GAGS are enzymatic products of proteoglycans formed by polysaccharide chains composed of repeating identical disaccharide units. GAGS have been found in the kidney, mainly in the papilla. Little is known about the mechanism whereby GAGS are excreted in urine [20]. In several models of experimental calcium oxalate nephrolithiasis, GAGS have been shown to inhibit crystal growth and aggregation [21]. Also, adherence of crystals to renal epithelial cells is inhibited by specific urinary anions such as glycosaminoglycans or NCs [22]. Michaelacci et al. have shown that stone formers, both adults and children, excreted lower levels of urinary GAGS as compared with normal subjects, independently of the metabolic disorder [23]. The very low concentration of GAGS that we found in patients and normal subjects from NE of Thailand suggests an important role for these macromolecules in nephrocalcinosis and stone formation. Authors in different countries have found marked reduction in GAGS in dRTA [9]. They considered this finding an indicator of damage in distal tubule and ureteral tissue.

We characterized Ca^{2+} binding mechanisms of four NC-isoforms by electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) spectrometric methods using vanadyl ion as a paramagnetic probe [24, 25, 26]. The results show all four isoforms bind four metal atoms per molecule by EPR technique. Isoforms A and B bound metal ions directly through acidic amino acid residues; however, ENDOR results indicated that metal ions in isoforms C and D are exposed to solvent water molecule and two water molecules are present in the inner coordination sphere of the metal ion. The presence of inner water molecules makes either ''strong'' or ''weak'' inhibitor.

We found similar properties of kidney stone inhibitor NC in the urine samples collected in NE of Thailand. dRTA and kidney-stone-forming adult patients excrete significantly more (C + D) isoforms than (A + B) ($p < 0.05$). Isoforms A and B show low dissociation constant to CaOx crystals and strong amphiphilicity, which suggests that these inhibitors have strong affinity to CaOx crystals and polarized hydrophilic and hydrophobic groups in a molecule. In contrast, C and D isoforms have weaker (at least 10-fold less compared with A and B isoforms) affinity to CaOx crystals. This finding of higher excretion of NC isoforms (C + D) in RTA and stone-forming patients compared with controls is also associated with nephrolithiasis in other regions that we have studied [15]. Recently we reported that NC isoforms cause different morphology of growing CaOx crystals by coating the surface of CaOX crystals [27]. It may be an indication that abnormal NC is one of the major factors responsible for the pathogenesis of nephrolithiasis in this region.

Our previous studies provided evidence that potassium deficiency exists in this endemic region [17]. Chronic potassium deficiency can lead to tubulointerstitial changes in several animal models [28, 29]. If such damage occurs in the proximal tubule (a site known to synthesize NC), it is possible to alter the structure of the cells and result in change of phenotypes of the synthesized proteins.

Lonsdale reported morbidity of human biomineralization, and analyzed nephrolithiasis in NE Thailand 40 years ago [30]. She stated that it was difficult to find a common denominator for the cause of this condition. We studied urine samples from the same area where Lonsdale made her observations and we believe that NC and GAGS are very important factors in the pathogenesis of this disease.

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