Urol Res (2001) 29: 89–93

ORIGINAL PAPER

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The reappraisal of nephrocalcin – its role in the inhibition of calcium oxalate crystal growth and interaction with divalent metal ions

Received: 20 June 2000 / Accepted: 16 November 2000

Abstract Nephrocalcin (NC) is a potent crystal growth inhibitor of calcium oxalate monohydrate. However, the sequence is undefined owing to its multiple glycosylations. Although there have been many biochemical studies of the binding capacity of calcium, the study of the function of the domain is still deferred. By using S-200 gel filtration and Mono Q ion-exchange chromatographic procedures, NC can be purified without further treatment for the removal of urobilirubin. The kinetic study of crystal growth in calcium oxalate monohydrate is performed using a seed slurry system. NC was cut into two peptides through interaction with copper ion and ascorbic acid. The interaction site of the copper ion is presumed to be located between 8 and 6 kDa of molecular weight in NC. The data suggest that divalent metal ions may be involved in the calcium oxalate crystallization through interaction with NC. The role of ascorbic acid in the formation of urinary stones should be reappraised for its association in the redox reaction, with resultant protein digestion in the presence of copper ions.

Key words Calcium oxalate monohydrate · Crystallization · Metal ion · Nephrocalcin · Urolithiasis

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Introduction

The processes of crystallization, such as nucleation, growth, and aggregation, are involved in urolithiasis. In an in vivo study, the rate of crystal aggregation was inhibited by urine [19]. Various urinary proteins have been suggested as promoters, inhibitors, or complexors in the above processes, which were supported by the fact that macromolecules participated in the formation of urolithiasis [5]. Although the macromolecules isolated from urine have been extensively studied all over the world, the exact roles of these proteins remain obscure. Urine contains numerous compounds that seem capable of inhibiting crystallization processes, but there are at present no data to show that a single compound is the sole most important inhibitor. Crystallization in urine is inhibited by a whole group of compounds that all contribute to the overall effect [15]. Therefore, studying the interaction of proteins with crystals in urine, could expand our understanding of the pathogenesis of urolithiasis.

Nephrocalcin (NC) is an acidic glycoprotein with abundant gamma-carboxyglutamic acid (GLA) residues. Nakagawa et al. isolated it in 1981 [11]. The physicochemical study of the properties of NC revealed it is a strong inhibitor of calcium oxalate monohydrate crystal growth and aggregation. A study using a ^{32}P-NMR spectrometer indicated that the four Ca^{2+} atoms saturate one molecule of NC [14]. Using a metal binding model from EPR and ENDOR, NC-A, which is one of the four isoforms of NC, is revealed as a strong inhibitor that is mostly isolated from healthy individuals. In contrast, NC-C, another isoform of NC, is a poor inhibitor, isolated from patients with recurrent stones [9]. Normal NCs are amphiphylic, which can be indicated by measuring the surface tension using a Lauda film balance [12]. Thus, we deduce that NCs are less water-soluble. This could inhibit further crystal growth of the hydrophilic sides attaching at the crystal and the hydrophobic sides facing outward [10]. However, the

sequences of the amino acids are still under investigation. Tang et al. showed that NC might be a urinary derivative (bikunin) of the inter-α-trypsin inhibitor [18]. Because of the large number of steps and the considerable amount of time needed for the isolation of NC, there have been few studies on the further mechanisms of stone formation within tubules, apart from the work by Nakagawa et al. [10]. This has delayed the identification of sequences and proteinomic studies of NC. However, when compared with the Tamm-Horsfall protein and uropontin, Coe et al. found that NC was a very potent calcium oxalate crystal inhibitor in vitro [2]. The primary aim of our study was to investigate a new method in the isolation of NC to facilitate further study of this protein.

There was evidence of trace metals involved in the inhibition of crystal growth through interaction with citric acid [8]. Iron, aluminum, and chromium ions were studied. However, other divalent ions were not included. Trace metal-citric acid complexes that have formed could be inhibitors of calcium oxalate crystal growth. However, the interaction of metal ions with urinary protein has not been thoroughly investigated. The second aim of this study is to investigate the interaction of divalent metal ions with NC.

Materials and methods

Materials

Sephacryl S-200 and Mono Q HR 5/5 columns were purchased from Pharmacia Biotechnology (Uppsala, Sweden). A fast protein liquid chromatography (FPLC) system was purchased from Pharmacia (Sweden). The high-performance liquid chromatography (HPLC) system LC-9A was purchased from Shimadzu (Japan). The spectrophotometer was purchased from Beckman (DU-640, Palo Alto, Calif., USA). The Hotech 631-D constant-temperature circulating bath was purchased from Hotech instruments (Taiwan). The stirred cell system and molecular filtration membrane of 5000 Da were obtained from Spectrum (Houston, Tex., USA). The electrophoresis gels and silver stain plus were purchased from Bio-Rad Laboratories (Hercules, Calif., USA). Reagents for determination of protein concentration (Bradford's method) were also purchased from Bio-Rad Laboratories. Gels and columns were recycled according to the manufacturer's instructions before the procedure. Low pre-stained molecular weight markers were purchased from Electran (Poole, England). All chemical reagents used were purchased at analytical grade from Merck (Darmstadt, Germany).

Methods

Collection of urine

A 24-h urine sample was collected from a healthy adult male volunteer without any history of urinary stones. Routine renal sonography was performed before the collection of urine, to rule out possible nephrolithiasis. The sample was kept at 4 °C during collection. Sodium azide (0.02% W/V) and 5 μM phenylmethylsulfonyl flouride (PMSF) were added as preservative agents. A test-strip was done to confirm the absence of blood. After collection, the sample was filtered through 0.22 μm filter membrane (Millipore, Bedford, Mass., USA) and stored at −70 °C until further procedure.

Ultra-filtration of the urine sample

The volume of urine sample was lessened to 1/10 in stirred cell system with 5 kDa filtration membrane. The procedure was performed at 4 °C under nitrogen gas pressure, according to the manufacturer's manual.

Purification of Nephrocalcin

According to the introduction by Nakagawa et al., NC can be purified using the procedures of dialysis, 0.4 M NaCl elute and three steps of column chromatography (DEAE, P-10, and S-200) [12]. In this study, we simplified the procedure by reducing it to two steps of chromatography using the FPLC system. Briefly, the concentrated sample was injected into the Sephacryl S-200 column (bed volume 200 ml) and protein was eluted with 0.02 M Tris, pH 7.3, buffer. The final step of purification was on the Mono Q 5/5 column using a linear gradient of NaCl solution from 0 M to 0.25 M, buffered with Tris 0.02 M, pH 7.3, in a FPLC system. The elute was incubated with 0.05 M EDTA, pH 7.9, and kept at 4 °C for 4 days [14]. After treatment, the solution was dialyzed against 12 l of dd water for 1 day with two changes.

Determining the concentration of NC

The method used to determine the concentration of protein eluted from chromatography was the Bradford assay. We added 800 μl of protein sample to separate test tubes. To each tube we added 200 μl of the Bio-Rad Dye Reagent Concentrate and a vortex to mix the sample well. After incubation for 5 min, the samples were transferred into cuvette. Using the spectrophotometer, the concentration of NC was obtained by the detection of 595 nmabsorbency. The data were corrected by a comparison with the standard solution of bovine serum albumin. The presence of urobilirubin was tested by adding 1 ml of sample into cuvette, and 400 nm/625 nm optical density was measured [12]. Reference protein was bovine serum albumin in 1 μM concentration.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed on 1-mm-thick 12% polyacrylamide gel using Bio-Rad Mini Protean II apparatus. Proteins and molecular-weight markers were electrophosed and subjected to the reduction in the presence of SDS and β-mercaptoethanol. The gel was stained using Bio-Rad silver stain plus.

High-performance liquid chromatography (HPLC)

Ten percent of trifluroacetic acid (TFA) was added to the sample and centrifuged with 6000*g* for 10 min. The supernatant was injected in a reverse-phase column (Waters, Bondapak TM, C-18, Milford, Mass., USA). The column was equilibrated with 10% TFA. The protein was then eluted in a 0–10% dimethylsulfoxide (DMSO) gradient.

Amino acid analysis

Samples were hydrolyzed in an evacuated sealed tube containing 6 N HCl at 110 °C for 24, 48, and 72 h. The hydrolysate was dissolved in sodium citrate buffer, pH 2.0, after removal of HCl in vacuo. Amino acid composition was determined with a Beckman amino acid analyzer (System 6300; Beckman Instruments, Palo Alto, Calif., USA). The amino acid composition is expressed as mole percent of amino acid residues.

NC and calcium oxalate inhibition

Calcium oxalate crystal growth inhibition activity was measured using the absorbance of the assay solution at 214 nm, using a spec-

trophotometer according to the previous report by Nakagawa et al. [13]. Purified monomeric NC was dissolved in water to a concentration between 10^{-6} and 10^{-8} M. Calcium oxalate monohydrate (COM) crystals were prepared as previously described by Nakagawa et al., and the product was verified using an infrared spectrometer (Bio-Rad, IR-7) [14]. The seed crystal slurry was made by suspending 8.0 mg of the solid COM crystals in 10 ml of deionized distilled (dd) water, equilibrating the mixture with stirring overnight, then bringing it to 37 °C for 1 h. The sodium oxalate (0.167 mM) and calcium chloride (0.833 mM) solution were equilibrated at 37 °C; a 1-ml aliquot of each was transferred to a cuvette at 37 °C using a constant-temperature circulating bath and magnetic stirring. When the baseline stabilized, an aliquot of NC (100 µl) was added to the assay solution. Crystal growth was initiated using the addition of 30-µl seed crystal slurry. The absorbance was monitored at 214 nm for 5 min. The experiments were performed in triplicate. The basic unit of protein concentration used in this study was 10^{-9} M. The inhibition of NC in the crystal growth was calculated thus:

$$\text{Inhibition}(\%) = Ao - Ai/Ao$$

Where *Ao* is the change of absorbance unit in the control group at 400 s. *Ai* represents the absorbance change in the group of NC. IC_{50} values were calculated from regression lines. *X* is the concentration of NC and *Y* represents the percentage of inhibition.

Interaction of NC with divalent metal ions

The divalent metal was dissolved in dd water with a concentration of 2.5 M for the addition. The volume in the addition of divalent metal in the cuvette was 5 µl. Selective divalent metal ions such as iron, copper, cobalt and manganese were used in this experiment. NC (100 mg) was put in 1 ml PIPES buffer (pH 5.8) and incubated with 100 mM divalent metal ions at 37 °C for the reduction of ions by 100 mM ascorbic acid. The solution was dialyzed against 3 changes of dd water for 48 h before being recovered. SDS-PAGE electrophoresis was performed after the incubation.

Results

Chromatography

It appears that macromolecules can be removed using Sephacryl S-200 gel filtration. The retention volume of 12–14 kDa molecules was 200–350 ml. The maximal inhibitory activity was distributed at 200–300 ml of elutes. Figure 1 depicts four waves of elutes from 0 to 250 mM NaCl gradient. Four waves were named A, B, C, and D; all of the waves had inhibitory activity. Elute A revealed conductivity of 5–9 ms/cm, then 9–13.5 ms/cm, 13.5–17.5 ms/cm, and 17.5–23 ms/cm were B, C and D, respectively. The protein used in the inhibitory assay was from elute A. Absorbances of 400 nm and 625 nm show diminishing in S-200 and Mono Q steps, which represent the presence of urobilirubin. The highest absorbance appeared at the completion of the ultrafiltration step. Urobilirubin was effectively removed in the final step (Table 1).

SDS-PAGE, HPLC, and amino acid compositions

HPLC was used to test the purity of NC after chromatography. It showed a single band of this elute, and the composition was confirmed and compared with the original report by Nakagawa et al. [13] (Table 2). There was over 24% composition of aspartic acid, and glutamic acid in the amino acids composition that was evidenced was NC. The protein was a relatively hydrophobic agent that was evidenced by the procedure of changing from TFA to DMSO. The protein was soon eluting out from the reverse-phase chromatography.

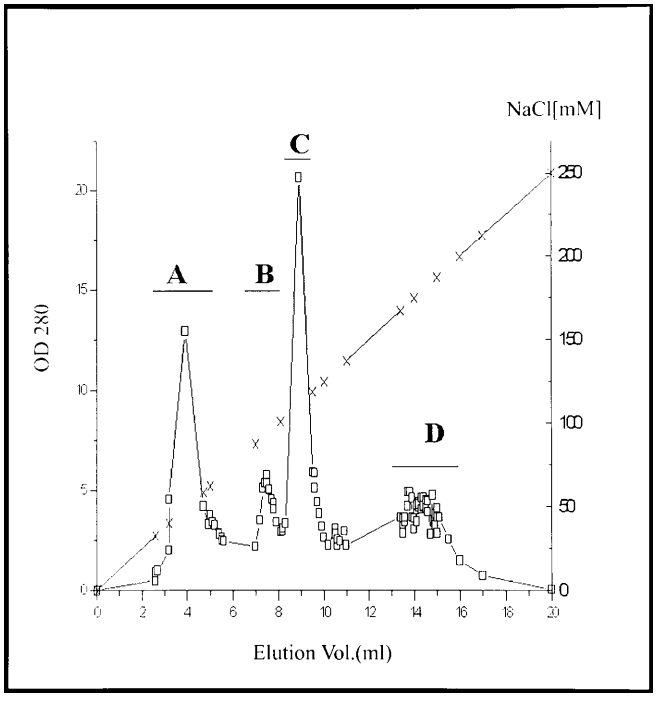

Fig. 1 Four fractions of elutes from 0 to 250 mM NaCl gradient through the column of ion-exchange chromatography (mono Q). Isoforms *A*, *B*, *C*, and *D* of NC showing inhibitory activity

Table 1 The optical density of 400 nm and 625 nm of the tested sample in the different steps of purification reveals a gradual decrease in the amount of urobilirubin. Each sample represented a 1-ml sample in the cuvette. The concentration of protein in the chromatographic step and reference protein – albumin for the test was 1 µM. Original 1-ml volume was used in the step of fresh urine and ultrafiltration

Step	OD: 400/625 nm
Fresh urine	0.52/0.14
Ultrafiltration	3.14/4.50
Sephacryl S-200-1	0.1265/0.0301
-2	0.2897/0.0736
Mono Q	0.0683/0.0046
Albumin	0.0387/0.0291

NC and calcium oxalate inhibition

The IC_{50} of NC revealed 284.6 nM by the calculation from linear regression, where $y = 0.1692x + 1.8367$ (Table 3). According to the Nakagawa's report, the IC_{50}

Table 2 Amino acid compositions of the fractions from Mono-Q chromatography. The data are listed in % of the whole amino acids in individual isoforms of nephrocalcin after being further eluted from HPLC (*ND* non-detected)

	A	B	C	D
Aspartic acid	13.2	15.3	16.4	15.8
Glutamic acid	19.3	18.4	18.3	18.3
Serine	7.6	3.3	3.5	3.9
Glycine	ND	16.9	15.4	12.3
Histidine	4.0	2.2	3.3	3.9
Arginine	3.8	3.6	4.2	4.4
Threonine	7.0	3.9	3.9	4.2
Alanine	7.1	3.9	5.5	5.9
Proline	5.7	4.0	3.0	3.3
Tyrosine	ND	3.7	3.8	3.8
Valine	4.4	2.8	4.1	4.2
Methionine	1.3	1.2	1.3	1.8
Cystine	11.7	4.8	4.6	4.7
Isoleucine	2.5	1.9	1.7	3.1
Leucine	4.2	3.2	3.3	5.2
Phenylalanine	2.7	3.2	2.5	2.4
Lysine	5.4	8.0	5.2	3.5
Tryptophan	ND	ND	ND	ND

Table 3 Percentage of inhibition and IC_{50} of Nephrocalin resulting from the crystal inhibitory assay. Each determination represents the mean average of triplicate determination

	10 nM	20 nM	50 nM	100 nM	IC_{50}
Nakagawa's report	16%		18%	24%	393.29 nM[a]
Present work	3.0%	6.0%	10.0%	18.2%	284.6 nM[b]

[a] $y = 0.0902x + 14.5250$
[b] $y = 0.1692x + 1.8637$

of NC was recalculated by linear regression, 393.29 nM was determined where $y = 0.0902x + 14.525$ [20].

Effect of divalent metal ions on NC

Based on the SDS-PAGE electrophoresis, NC was cut into two peptides. In the process, NC interacted with copper ions and ascorbic acid (Fig. 2). This showed the interaction site of the copper ion, which was located between 8 and 6 kDa of molecular weight in NC. In this biochemical study, copper did not influence the behavior of NC at normal physiological concentrations.

Discussion

Macromolecules in urine that have influenced the growth of COM crystals have been shown in several reports. However, the interaction between macromolecules and crystal surfaces in vivo is a complex phenomenon influenced by such physiological parameters as pH, ionic strength, and inorganic ion concentration [17]. Since the inhibition assay is primary in an ideal chemical condition and the concentrations used of ascorbic acid

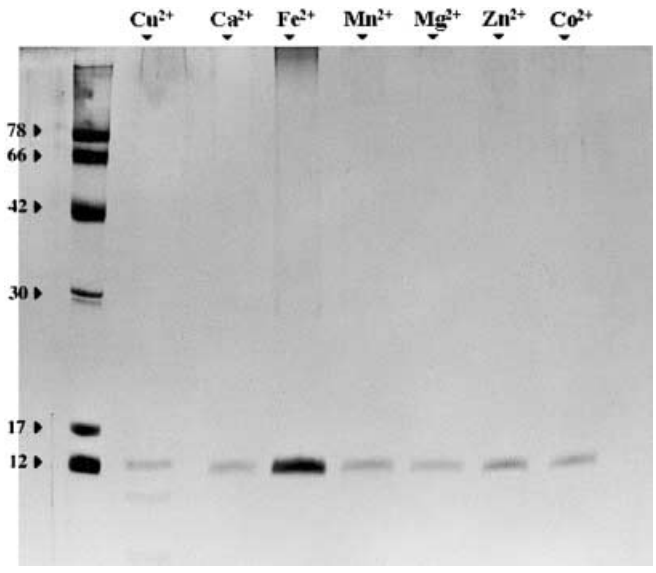

Fig. 2 Electrophoresis of nephrocalcin treated with various metal ions and ascorbic acid. *Lane 1*: Molecular weight marker. *Lanes 2–7*: Nephrocalcin that interacted with divalent ions, through reducing reaction, is shown on SDS-PAGE. *Lane 7*: An additional two relatively low molecular weight bands are shown below the original band of nephrocalcin

and metal ions were higher than the expected true urinary condition, it did not completely fulfill real urinary conditions. The evaluation of inhibitory activity of individual macromolecules, such as NC should further mimic the complex conditions of urine instead of a pure chemical status. This was a preliminary result that should be further explored in future studies on interactions between NC and urinary components.

Although there have been some clinical studies concerning the inhibitory activity of NC, there were fewer catalytic experiments for NC. The first experiment was used in the binding activity of calcium, which competes with other divalent metal ions. Four divalent metal ions interacted with NC through allosteric binding that resulted in the increasing inhibitory activity of NC. It showed that copper interacted with NC to form a complex, causing massive binding of calcium with NC. Overall, there was evidence of Mn, Co, and Fe in the binding with NC. Copper was less evident, but competing inhibition with other metal ions may be considered.

Ascorbic acid has long been discussed as the cause of urinary stone formation [3]. It has been proposed that ascorbic acid transforms into oxalate in the liver and is subsequently responsible for 40% of urinary oxalate [1]. However, ascorbic acid can be excreted in urine after a certain amount of oral intake and appear to modulate directly into calcium oxalate crystallization in the stone former [16]. Instead of being transformed into oxalate, ascorbic acid has some roles in urine. Nevertheless, ascorbic acid is soluble in urine and is a strong reducing agent that reduces copper [7]. A reversible, reducing copper ion would transport electrons and damage proteins, like NC. Using this possible prediction, we

investigated the hypothesis that NC was fragmented into two peptides and lost the function in vitro. There is evidence that high concentrations of ascorbic acid in the presence of copper ions will attack NC and cause calcium deposition in a urinary environment. Examples are hypercalciuria and nephrocalcinosis in patients with Wilson's disease [6]. Besides the safe doses of ascorbic acid, patients with a high concentration of urinary copper ions and the original form of ascorbic acid in excretion should be further investigated clinically for possible stone formation. Synergistic interactions of proteins and urinary composition might be important factors for the inhibitory activity toward calcium oxalate crystallization. Furthermore, Durak and co-workers reported that copper, zinc, and iron elements in the stone nucleus were significantly higher than the rest part of stone, suggesting a complex relationship between the elements of the urinary calculi [4].

In the kinetic study of divalent metal ions, copper was added, which showed an inhibitory activity compatible with the enzymatic Michaelis-menton model. We suggest that there may be a binding site for copper in NC, other than the calcium binding site. The binding increased the inhibitory activity of NC. It suggests that further study of the copper binding site and of amino acid residue, through peptide mapping, is urgent. It will prove that NC has a double metal ion-binding site that may be an allosteric interaction.

To summarize, we have presented an alternative method for the purification of NC. The methods previously used can be simplified by reducing the procedure to two steps without further treatment to remove urobilirubin. The inhibition of crystallization by biomolecules such as NC, Tamm-Horsfall protein, and albumin, should be further studied for the interactions of urinary proteins or metal ions. Because NC is a potent inhibitor of calcium oxalate crystallization in urine, further study of the molecular structure and its reactions should be undertaken to investigate the detailed mechanism of inhibition.

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