Is nephrocalcin a membrane-bound protein?
A biochemical generate hypothesis

**Abstract**

Introduction: Nephrocalcin (NC) is a urinary glycoprotein that plays an important role in the inhibition of calcium oxalate crystallization. NC occurs in at least four isoforms (NC-A, -B, -C, and -D). NC isoforms are phosphoproteins, but the abnormally high phosphate residues, particularly in NC-C and NC-D, are intriguing. It is possible that NC molecules could coat the surface of renal epithelial cells for preventing attachment of calcium oxalate crystals, and could conjugate membrane phospholipids (PL). Our objective was to identify and characterize PL in NC.

Material and Methods: NC and its four isoforms were isolated from non-stone forming urine. Phosphate contents were determined. Crystal growth inhibition rate of NC toward calcium oxalate monohydrate crystals (COM) was measured by decrease of oxalic acid. Phase separation of lipids and PL from proteins was carried out by a phase separation method using Triton X-114.

Results: The following compounds were identified: phosphatidylcholine, phosphoethanolamine, phosphatidylinositol, triglycerides, and neutral lipids. Amino acid composition of the four NC isoforms before and after extraction did not change. After Triton X-114 extraction, phosphate residues in isoforms B, C, and D were decreased nearly 1/10 of the original value. However, isoform A phosphate residues showed no change. Dissociation constant toward calcium oxalate was not significantly altered.

Conclusions: These data indicate the presence of typical membrane PL in NC molecule. NC, including all its isoforms, could be a membrane bound protein, conjugating membrane PL and coating the surface of the epithelial renal cells.

Keywords: kidney stone, calcium oxalate, kidney stone inhibitor, nephrocalcin, nephrolithiasis.

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**Introduction**

Nephrocalcin (NC) is a glycoprotein that plays an important role in the inhibition of urinary crystallization. NC affects the habit, size and crystal structure of calcium oxalate crystals. Indeed, NC controls conversion of calcium oxalate monohydrate crystals (non-octahedral) into small cubic octahedral crystals, which are more characteristic of calcium oxalate dihydrate.

NC occurs in many polymeric forms with molecular weights from 14 to 68 kDa, and it elutes from DEAE-cellulose in at least four NC isoforms (NC-A, -B, -C, and -D). There is a strong correlation between patients’ probabilities of suffering kidney stones and the proportions of the different isoforms in their urine. People who are more likely to generate kidney stones excrete greater proportions of isoforms NC-C and -D than NC-A and -B. Like the urine of stone formers, extracts of surgically removed kidney stones that were composed of over 60% calcium oxalate also exhibited more of NC-C and -D than -A and -B.

NC-A and -B exhibit different biochemical and functional properties compared to NC-C and -D. In subjects with nephrolithiasis, dissociation constants of NC-A and -B for calcium oxalate crystals are approximately \(1 \times 10^{-7}\) M, but those of NC-C and -D are only 1 to \(10 \times 10^{-6}\) M, indicating that NC-A and -B bind those crystals at least tenfold more tightly than do NC-C and -D. Circular dichroism spectra show that NC-A and -B change conformation upon binding calcium, although -C and -D do not.

NC isoforms contain high amount of phosphate. Interestingly, when we investigated NC-C phosphorylation and glycosylation using P31-NMR, we found some phosphorylation that occurred at serine – OH group. Dephosphorylation by alkaline phosphatase...
and chemical deglycosylation decreased phosphate content, but a substantial amount of phosphate remained on the molecule. We assumed that NC isoforms are phosphoproteins; however, the abnormally high phosphate residues, particularly in NC-C and NC-D, are intriguing. We then hypothesized that NC molecules could coat the surface of inner proximal tubules for preventing attachment of calcium oxalate crystals,\textsuperscript{9,10} and in this situation could conjugate membrane phospholipids. 

Our objective in this work was to identify and characterize possible phospholipids in NC using phase separation methods.

**Materials and Methods**

Nephrocalcin and its four isoforms were purified from a 24-hour urine sample obtained from a healthy male without personal history of stone disease, known illness, or use of any chronic medication as described previously.\textsuperscript{6,11} Amino acid analysis was performed after it was hydrolyzed in a sealed tube containing 6 NHCl at 110°C for 24 hrs, and subjected to a Beckman amino acid analyzer model 118C.

Phosphate contents were determined by the method reported by Ames and Dubin.\textsuperscript{12} Briefly, a NC sample was digested with 60 µL of 10% Mg(NO\textsubscript{3})\textsubscript{2} in ethanol over an open flame, followed by 1 NHCl hydrolysis in a boiling water for 15 minutes, then it developed a color by adding ammonium molybdate/ascorbic reagent. The calibration curve was prepared using 0.01 MKH\textsubscript{2}PO\textsubscript{4} as a stock standard solution by measuring absorbance at 820 nm. The spectrophotometer used was a Beckman DU 640.

Crystal growth inhibition rate of NC toward calcium oxalate monohydrate crystals (COM) was measured by decrease of oxalic acid at 214 nm in the presence of seed crystals at pH 5.8 using a spectrophotometer connected to a computer for determination of second order reaction rate, as reported previously.\textsuperscript{5} Dissociation constant was calculated from the slope of the Langmuir isotherm type plot.\textsuperscript{5}

The separation phase of lipids and phospholipids from proteins was carried out by a phase separation method using Triton X-114, as reported by Bordier.\textsuperscript{13} NC, 500 µL, in 0.02 M Tris-HCl, pH 7.2 containing 0.2 M NaCl, was incubated with 100 µL of pre-condensed Triton X-114 at 4°C for 20 minutes, then transferred to a 37°C incubator for 30 minutes. The cloudy mixture was centrifuged at 7,000 x g for 10 minutes, and the bottom layer (Triton X-114 fraction) and the top layer (aqueous protein fraction) were separated.

Thin layer chromatography was carried out by a silica gel G plate (20×20 cm). The silica gel G plate (Analtech Inc, Newark, DE, USA) used had 70 Aº pore size in average and coated 0.25 mm thickness on a glass plate. The plate was activated by heating at 100°C for 30 minutes in an oven before use. Samples were developed in two dimensions: chloroform/methanol/28% ammonia (65/35/5, v/v/v) for the first dimension, and chloroform/methanol/acetic acid/water (20/10/40/10/5, v/v/v/v/v) for the second dimension. All solvents used were HPLC grade and obtained from Fischer Scientific Co. The developed chromatogram was examined by two methods. First, the plate was exposed to iodine vapor and spots were recorded. After removing iodine vapor, 0.0012% Rhodamine 6G sprayed aqueous solution was added, and fluorescent spots were observed under 365 nm UV lamp.

Derivatization of fatty acids was achieved by using 9-anthryldiazo methane (ADAM) reagent. ADAM was synthesized from 9-anthraldehyde and hydrazine hydrate in ethanol by following the method described by Nakaya.\textsuperscript{14} An aliquot of 200 µL of standard fatty acids solution, or Triton X-114 fraction in methanol, was mixed with 400 µL of ADAM (6.3 mg/mL of aceton) and kept in dark at room temperature for overnight. The following standard lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA): phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, cholesterol, decanoic acid, lauric acid, palmitic acid, stearic acid, and linolenic acid. Derivatized lipids were analyzed by a Beckman HPLC system (166 detector and 125 solvent module) using C18 reversed phase column (Water Bondapk C18, 2×300 mm). The running conditions were: solvent system, methanol/water (47/53, v/v) isocratic mode, flow rate 0.5 mL/min, and detection wavelength at 210 nm.

**Results**

After the separation phase, the majority of the proteic component of NC was found in an aqueous layer, and lipids and phospholipids remained in Triton X-114 phase. Lipid and phospholipid composition in Triton X-114 fractions was examined by TLC, and a chromatogram was developed. In this method, acidic phosphatides and other lipids show blue or purple fluorescence, and neutral lipids and neutral phosphatides give yellow or orange color under UV light (366 nm). According to these colors and Rf values comparing with standards, the following compounds were identified: phosphatidylcholine, phosphoethanolamine, phosphatidylinositol, triglycerides, and neutral lipids. Next, these Triton X-114 extracts were modified by ADAM and subjected to HPLC analyses. The following fatty acids were identified from retention time: C10 (decanoic acid), C12 (lauric acid), C16-1 (palmitoleic acid), C-18 (stearic acid), C18-1 (oleic acid), C18-2 (linoleic acid), and C18-3 (linolenic acid).
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Amino acid composition of the four NC isoforms before and after Triton X-114 extraction (aqueous layer) did not change. All amino acid residues were the same within narrow experimental margin of error before and after the detergent extraction.

Phosphate content was compared before and after Triton X-114 extraction, and the results are shown in Table 1. NC isoforms C and D contained more phosphate residues compared to isoforms A and B. After Triton X-114 extraction, phosphate residues in isoforms B, C, and D were decreased nearly 1/10 of the original value. However, isoform A phosphate residues showed no change.

Dissociation constant toward COM is shown in Table 1. Isoforms A and B showed no differences before and after phosphate removal. The values of isoforms C and D decreased one order, which indicate a slight decrease in affinity toward COM compared to isoforms A and B.

**DISCUSSION**

In this work, we were able to show that NC has a significant amount of phosphate, composed in part by several phospholipids. One possible explanation to this finding could be anchorage of NC in renal epithelial tubular cells and posterior incorporation of these membrane phospholipids in its molecule.

Some macromolecules present in the urine can promote or inhibit urinary crystallization depending of the conditions prevalent in the urine. Tamm-Horsfall protein (THP), for example, has been implicated in stone formation, inhibiting calcium oxalate crystal aggregation. However, conditions of high ionic strength in the urine and low urinary pH favor THP self-aggregation and crystal formation. THP in solution can behave differently than when it is attached or adsorbed to a surface. Rindler et al., using the same Triton X-114 partitioning assay that we used here, were able to demonstrate that THP has a hydrophobic character when attached to cellular membranes, in contrast to its hydrophilicity after isolation from human urine. In addition, they showed that THP is a member of glycosylphosphatidylinositol (GPI) class of lipid-linked membrane proteins and it is released into the urine after losing its hydrophobic anchor, probably due to the action of a phospholipase or protease.

In this work, the Triton X-114 separation phase was used without denaturing the protein, and we found the presence of phospholipids, lipids and fatty acids in the NC molecule. From two dimension TLC and chemical modification of fatty acids followed by HPLC separation, we identified lipids and phospholipids that are typical constituents of cell membranes. Judging from the lipid compositions in this report (linoleic, linolenic, oleic and palmitoleic acid, phosphoethanolamine, phosphatidylinositol, stearic acid) and carbohydrate compositions that we previously reported (galactose, glucose, glucosamine, galactosamine, mannose, and N-acetyl-neuraminic acid), NC isoforms could be a cell surface anchored protein through GPI.

Urinary glycoproteins are important inhibitors of calcium oxalate crystallization and adhesion of crystals to renal cells, both of which are key mechanisms in nephrolithiasis. Multiple anchored and soluble renal proteins modulate or even regulate these events. Lieske et al., using renal epithelial cells (BSC-1 line) demonstrated

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**Table 1**

<table>
<thead>
<tr>
<th>Original (before extraction)</th>
<th>PO₄₄ nmole/mg NC</th>
<th>Dissociation constant (kd)</th>
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<tbody>
<tr>
<td>NC isoform</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.08</td>
<td>4.29 × 10⁻⁷ M</td>
</tr>
<tr>
<td>B</td>
<td>23.06</td>
<td>2.65 × 10⁻⁷</td>
</tr>
<tr>
<td>C</td>
<td>315.6</td>
<td>4.38 × 10⁻⁷</td>
</tr>
<tr>
<td>D</td>
<td>795.1</td>
<td>1.86 × 10⁻⁷</td>
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</table>

<table>
<thead>
<tr>
<th>After Triton X-114 extraction</th>
<th>PO₄₄ nmole/mg NC</th>
<th>Dissociation constant (kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A’</td>
<td>1.14</td>
<td>8.55 × 10⁻⁷ M</td>
</tr>
<tr>
<td>B’</td>
<td>5.44</td>
<td>1.90 × 10⁻⁷</td>
</tr>
<tr>
<td>C’</td>
<td>34.9</td>
<td>1.08 × 10⁻⁶</td>
</tr>
<tr>
<td>D’</td>
<td>78.3</td>
<td>0.72 × 10⁻⁶</td>
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that nephrocalcin, uropontin and citrate inhibited binding of calcium oxalate crystals. We postulated that NC present in tubular fluid and on the surface of epithelial tubular renal cells could help determining whether a crystal-cell interaction would result in retention of the crystal or its passage out of the nephron. Our results also suggest that phospholipids and lipids found in the formed calcium oxalate kidney stones were rather from matrix proteins, and those lipids have no direct function to kidney stone formation.

NC-A isoform was lipid-free, but B, C, and D carried lipids and phospholipids, as showed in Table 1. When these lipids were separated from NC molecules, dissociation constant toward calcium oxalate from NC-C and D just showed a small variation (from 10-7 to 10-6 M), and amino acid composition was not changed. This observation indicates that lipids and phospholipids are not absolutely necessary for NC calcium oxalate binding. These data are in agreement with our previous studies that indicated all four isomers bound 4 atoms of Ca directly or indirectly to carbosyl groups of amino acids.

CONCLUSIONS

The natural defense against nephrolithiasis may include impeding crystal attachment by an effect of macromolecular inhibitors like nephrocalcin. Our data in this work suggest that the high amount and the type of phospholipids found in the molecule of NC could be acquired from interaction of NC with epithelial renal tubular cells. NC, including all its isoforms, could be a membrane bound protein coating the surface of the epithelial renal cells.

REFERENCES