Anomalous Phospholipid n-6 Polyunsaturated Fatty Acid Composition in Idiopathic Calcium Nephrolithiasis[1]

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ABSTRACT

Anomalies in the erythrocyte transport of anions and cations have been described in idiopathic calcium oxalate nephrolithiasis and seem to play a pathogenetic role in this disease. In consideration of the hypothesis that the complex array of ion flux cell abnormalities is an epiphenomenon of an anomaly in the composition of cell membranes, this study investigated cell-membrane lipid composition. In idiopathic calcium oxalate renal stone formers, in which ion transport abnormalities were present, and in healthy control subjects, plasma and erythrocyte membrane lipid composition, the erythrocyte oxalate exchange, and Na/K/2Cl cotransport activity were evaluated. Furthermore, in stone formers, the effect of a 30-day fish-oil diet supplementation on plasma lipids, erythrocyte oxalate exchange, oxaluria, and calciuria was investigated. The effect of arachidonic acid released by phospholipase A_2 on anion-carrier phosphorylation and activity in erythrocytes was evaluated as well. Patients had a lower content of linoleic and higher concentration of arachidonic acids in both plasma and erythrocyte membrane phospholipids, and an increased arachidonic/linoleic acid ratio. The arachidonic acid level correlated with the erythrocyte oxalate exchange and sodium cotransport activity. Fish-oil supplementation lowered calcium and oxalate urine excretion, and normalized the erythrocyte oxalate exchange. Phospholipase A_2 increased the erythrocyte anion-carrier protein phosphorylation and the oxalate exchange. This study shows that idiopathic calcium nephrolithiasis in the patient group reported here is characterized by a systemic defect in phospholipid arachidonic acid levels that might provide an answer to the link between genetic background, dietary habits, and renal lithiasis.

Key Words: *Renal stone, ion transport, hypercalciuria, hyperoxaluria, arachidonic acid*

Anomalies in the cellular transport of oxalate, urate, sodium, and calcium have been observed in erythrocytes from patients with idiopathic calcium nephrolithiasis (ICN) (1–5). At least some of these ion transport defects seem to be genetically controlled, and several findings suggest that they are also present at the intestinal and renal level, thus supporting their possible pathogenetic role in nephrolithiasis (2,3,5,6). These observations led us to consider calcium nephrolithiasis as a cellular disease characterized by a systemic defect of ion exchange, at least in a portion of ICN patients.

However, at least two points were still difficult to understand: (*1*) the unlikelihood that so many defects would be determined separately; and (*2*) the recognition of differences in carrier protein isoforms between different tissues (in the kidney and the gut) and red blood cells (RBC) precluded the assumption that a carrier that is structurally abnormal in RBC would be correspondingly structurally abnormal in the kidney, for example. Therefore, it is intriguing to explain the systemic distribution of the ion transport defects. We favor the idea that the complex array of ion-flux cell abnormalities observed in ICN is an epiphenomenon of a still unknown primitive anomaly, possibly in the composition of cell membranes, the milieu in which carrier proteins work.

This hypothesis was explored in reference to phospholipid metabolism in the light of the following considerations: (*1*) phospholipids are recognized as second cell messengers, and could influence membrane properties and membrane protein functions (7–9); (*2*) an alteration in the urine excretion of prostaglandin E_2 (PGE_2), a phospholipid metabolite, has been described in calcium nephrolithiasis (10–12); (*3*) dietary n-3 polyunsaturated fatty acids, which have been shown to be potent modulators of tissue phospholipid fatty acid composition and eicosanoid production *in vitro* and *in vivo* (13,14), reduce urine calcium and oxalate excretion (15,16); (*4*) nephrolithiasis is rare in Eskimos (17).

Here we report evidence of an anomalous arachidonic acid content in the plasma and erythrocyte membrane phospholipids of renal calcium stone patients. Experimental findings from a dietary interven-

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tional trial with fish oil and an *in vitro* study with phospholipase A_2 support an important role of this anomaly in the pathogenesis of calcium nephrolithiasis.

METHODS

Study Protocol

We studied 24 recurrent idiopathic calcium oxalate stone formers (11 females) (median, 32 yr of age; range, 21 to 60 yr) randomly selected among those attending our outpatient clinic. The diagnosis of idiopathic calcium nephrolithiasis was formulated according to previously described criteria (2). Eighteen healthy staff members (9 females) (median, 30 yr of age; range, 24 to 50 yr) were considered as control subjects; none had a family or personal history of nephrolithiasis, nor the oxalate transport defect. All patients and control subjects had normal renal function, sterile urine samples, and normal blood pressure; none had diabetes mellitus, nor hepatic, thyroid, or immunological diseases. All gave their informed consent to the study, according to the Helsinki Declaration. No patient or control subject had been placed on any type of therapeutic regimen in the 8 wk before the study, and in particular, none were taking any lipid-lowering drug or other agent known to affect lipid metabolism. During the 8 wk before, and throughout the 30-day study period, all subjects followed an isocaloric diet providing 55% of the total calories as complex carbohydrates, 20% as proteins, and 20% as fats (7% of which as saturated fatty acids, 10% as monounsaturated, 8% as polyunsaturated) with a cholesterol content of less than 500 mg/day. This is a standard diet of our region, and furnishes approximately 75 mg of oxalate, 800 mg of calcium, 65 mg of purine, and 5 g of sodium daily. Patients were asked to maintain their daily energy consumption and macronutrient distribution throughout the study period. At the beginning of the study, plasma lipid composition was evaluated in all subjects, whereas erythrocyte membrane lipid composition was determined only in ten randomly selected healthy people and ten randomly selected stone formers. In these control subjects and patients, the oxalate self-exchange rate and the Na/K/2Cl erythrocyte cotransport activity, the 24-h urinary excretion of oxalate, calcium, and PGE_2 were also determined (data previously reported in part) (5,18); the Na/K/2Cl K_m values, determined as previously reported (5), were 24.14 ± 11.08 mmol of Na^+/L of cells and 13.25 ± 4.20 mmol of Na^+/L of cells in stone formers and control subjects, respectively ($P = 0.01$); urine PGE_2 values were 248 ± 139 pg/min in ICN patients, and 123 ± 30 pg/min in control subjects ($P = 0.017$).

Seven out of these latter stone formers agreed to continue the study and received 1 capsule, three times daily, containing 850 mg of n-3 polyunsaturated fatty acid ethyl esters (fish oil) for 30 days. Each capsule contained 51.5 ± 0.6% and 39.1 ± 1.2% of fatty acids as C20:5(n-3) and C22:6(n-3) respectively. Other minor fatty acid components were C18:4(n-3) (2.3 ± 0.1%), C20:4(n-6) (3.0 ± 0.2%), C21:5(n-3) (1.6 ± 0.2%), and C22:5(n-3) (2.4 ± 0.6%). At the end of the 30-day n-3 fatty acid supplementation period, plasma lipid composition, the erythrocyte oxalate self-exchange rate, and the 24-h urinary excretion rates of oxalate and calcium were reevaluated in ICN patients.

Urinary Parameters

Urinary calcium and oxalate were evaluated on two consecutive 24-h urine samples; the results are reported as the mean of two values. Calcium was assayed by atomic absorption spectrometry. Oxalate was assayed enzymatically (oxalate oxidase; Sigma, St. Louis, MO).

Measurements of RBC Oxalate Exchange

The RBC oxalate self-exchange was assayed as previously described (2) and was expressed as K, an index of the oxalate flux at equilibrium, calculated from the slope of the linear relation:

$$\ln (A_t - A_\infty)/(A_0 - A_\infty) = -Kt,$$

where K denotes the flux rate, and A the quantitative of radioactive oxalate at Time 0, at Time t, and at isotope equilibrium (∞).

Lipid Analyses

Blood samples were collected from the antecubital vein after an overnight fast in vacutainer tubes containing EDTA. The plasma was removed by centrifugation at 3,000 × g for 10 min. Plasma lipids were extracted using chloroform-methanol (2:1) as described by Folch *et al.* (19). The RBC were washed three times by resuspension in five volumes of 0.15 mol of sodium chloride per L, and 0.1 mmol of EDTA per L (pH 7.4); 2 mL of packed erythrocytes were then lysed in 38 mL of a ice-cold buffer containing 10 mmol/L of Tris-HCl and 2 mmol/L of EDTA (pH 7.5), and washed five times with the same solution. Lipids were extracted from RBC membranes according to Rose and Oklander (20). Erythrocyte and plasma phospholipids were isolated by applying the lipid extract to a column of silica coupled with aminopropyl groups (Bond Elute NH_2; Analytichem International, Harbor City, CA). After transmethylation according to the method of Morrison and Smith (21), fatty acids were determined by gas chromatography with a Perkin Elmer 8320 gas chromatograph, equipped with a 30-m capillary column (0.32 mm internal diameter) (Omegawax 320; Supelco, Bellefonte, PA). Column conditions were 200°C, injection port 250°C, and flame ionization detector 260°C. Helium was used as carrier gas (linear velocity, 25 cm/s); the split ratio was 100:1. Fatty acid peaks were identified by comparison with mixtures of fatty acids (PUFA 1 and PUFA 2) supplied by Supelco. The amounts of individual fatty acids were calculated as a relative percentage, with the evaluated fatty acids as 100%. An aliquot of the lipid extract from erythrocyte membranes was used to measure different phospholipids. The phospholipids were separated by bidimensional thin-layer chromatography on 20 × 20-cm glass plates coated with a 500 μ layer of Silica Gel G (Alltech, Deerfield, IL). The first developing system was chloroform/methanol/water/NH_4OH (90:45:5.5:5.5), and the second was chloroform/methanol//water/acetic acid (90:40:2:12). Phospholipids were visualized with iodine vapor, and identified by comparison with standards (Supelco). Phosphate analysis of the spots corresponding to the different phospholipids (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylserine, phosphatidylinositol) was performed according to Bartlett (22). Cholesterol was measured by an enzymatic method (Monotest kit; Boehringer Mannheim, Mannheim, Germany), and proteins by the Lowry procedure (23).

Phospholipase A_2 Experiment

Human erythrocytes were prepared by centrifugation (at 750 × g for 3 min) of fresh blood from five healthy adult volunteers. To minimize contamination by leukocytes and

platelets, the packed RBC were washed three times by centrifugation in a buffer containing 20 mmol/L of Tris-HCl (pH 8.2), 140 mmol/L of sodium chloride, 10 mmol/L of potassium chloride, 1 mmol/L of magnesium chloride, 1 mmol/L of calcium chloride, 24 mmol/L of glucose, 1 mmol/L of adenosine, 100 μg/mL of streptomycin, and 25 μg/mL of chloramphenicol. Samples of packed RBC (0.5 mL), resuspended in 2 mL of the buffer A (20% hematocrit) were incubated in the presence or absence of 5 U phospholipase A_2 (*Naja naja atra*) (Calbiochem, San Diego, CA) at 300°C for 30 min. After incubation, the samples were washed once in the same buffer and subdivided into two aliquots; one was utilized to determine oxalate self-exchange as previously described (2), whereas the second was hemolyzed and processed to recover membranes as reported (24). Endogenous Ser/Thr-phosphorylation of membrane proteins, casein kinase activity in the isolated cell membranes, and autoradiography of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) slab gels were performed as described (24).

Statistical Analysis

Statistical analysis was carried out with the *t* test for paired and unpaired variables, and determination of the *r* coefficient for linear correlation.

RESULTS

Plasma and Erythrocyte Membrane Phospholipid Fatty Acid Composition

Plasma lipid values in patients were not significantly different from control levels (Table 1). There was also no significant difference in the percentage content in phospholipids of the RBC membranes between patients and control subjects (Table 2). In addition, the cholesterol/protein and phospholipid/protein ratios in patients were not significantly different from those in control subjects (data not reported). No difference was observed in phospholipid n-3 fatty acid content (percentage of total fatty acids), although an anomaly in the pathway of n-6 fatty acids was documented; in particular, patients had a significantly higher content of arachidonic acid in both plasma and RBC membrane phospholipids compared with healthy subjects ($P = 0.034$ and $P = 0.011$, respectively), a lower content of linoleic acid in plasma phospholipids ($P = 0.028$) (Table 3), and a higher arachidonic/linoleic acid ratio in plasma phospholipids (0.48 ± 0.13 *versus* 0.38 ± 0.08; $P = 0.011$).

A direct correlation between the plasma and erythrocyte membrane levels of arachidonic acid in phospholipids was observed in patients ($P = 0.033$) (Figure 1). Furthermore, the arachidonic acid content in RBC membrane phospholipids significantly correlated with transmembrane oxalate transport ($P = 0.019$) (Figure 2) and to the Na/K/2Cl cotransport K_m ($P = 0.023$) (Figure 3).

TABLE 1. Plasma lipids[a]

Plasma Lipids (mg/dL)	Patients ($N = 24$)	Control Subjects ($N = 18$)
Cholesterol	188.3 ± 35.7	204.5 ± 27.0
Triglycerides	89.9 ± 34.0	83.9 ± 26.3
HDL cholesterol	55.9 ± 15.4	57.8 ± 13.4
Phospholipids	199.1 ± 25.2	209.0 ± 29.6

[a] Data are presented as mean ± SD.

TABLE 2. Percentage of phospholipid content of the RBC membranes[a]

Phospholipid	Patients ($N = 10$)	Control Subjects ($N = 10$)
Phosphatidylcholine	30.56 ± 4.34	30.75 ± 2.38
Phosphatidylserine	10.39 ± 1.63	11.79 ± 2.90
Phosphatidylethanolamine	30.27 ± 4.19	29.45 ± 1.57
Sphingomyelin	28.47 ± 1.43	27.50 ± 1.69
Phosphatidylinositol	0.61 ± 0.46	0.74 ± 0.34

[a] Data are presented as mean ± SD.

Fish-Oil Dietary Supplementation Trial

Plasma lipid levels and the percentage of fatty acids in the plasma phospholipids in seven patients before and after 30 days of n-3 polyunsaturated fatty acid supplementation are reported in Tables 4 and 5. As expected, and also confirming good dietary compliance, this regimen induced a reduction in the plasma phospholipid content of linoleic ($P = 0.017$), eicosatrienoic ($P = 0.016$), and arachidonic acids ($P = 0.002$), and an increase in eicosapentaenoic ($P = 0.022$), and docosahexaenoic acids ($P = 0.018$). After n-3 fatty acid supplementation in stone formers, urinary calcium and oxalate excretion was significantly reduced ($P = 0.022$ and $P = 0.015$, respectively) (Figure 4 and 5). Moreover, RBC transmembrane oxalate self-exchange was normalized ($P = 0.000$) (Figure 6).

Effect of Phospholipase A_2 on the Erythrocyte Anion Carrier

When added to intact RBC, phospholipase A_2, which releases arachidonic acid from membrane phospholipids, determined a significant increase in ^{32}P incorporation in band 2 and 3 membrane proteins (+50.0 ± 9.6%, $t = 11.5$, $P = 0.000$; and +20.0 ± 8.9%, $t = 5.09$, $P = 0.007$, respectively), the activation of casein kinase (+42.6 ± 13.4%, $t = 7.07$, $P = 0.002$) (Figure 7), and a parallel increase in RBC oxalate self-exchange (+33.0 ± 14.5%, $t = 4.94$, $P = 0.008$).

DISCUSSION

The major finding of this study was an increased content of arachidonic acid in phospholipids in plasma from ICN patients; all other lipid values were not different from those of control subjects (Tables 1, 2, 3). A similar alteration in arachidonic acid content is also present in RBC membrane phospholipids (Table 3), and was directly correlated with the plasma

TABLE 3. Percentage of fatty acids in phospholipids[a]

Fatty Acid	Plasma		Red Blood Cell Membranes	
	Patients	Control Subjects	Patients	Control Subjects
	(*N* = 24)	(*N* = 18)	(*N* = 10)	(*N* = 10)
C14:0 (myristic)	0.6 ± 0.2	0.7 ± 0.2	1.1 ± 0.4	1.1 ± 0.4
C16:0 (palmitic)	29.7 ± 2.7	28.9 ± 2.2	28.5 ± 2.8	26.7 ± 1.6
C18:0 (stearic)	13.6 ± 1.4	12.9 ± 1.2	10.6 ± 1.2	11.4 ± 2.1
C24:0 (lignoceric)	0.2 ± 0.1	0.4 ± 0.4	0.6 ± 0.5	0.7 ± 0.2
C16:1n-7 (palmitoleic)	0.9 ± 0.3	1.0 ± 0.3	0.93 ± 0.51	0.91 ± 0.29
C18:1n-9 (oleic)	12.3 ± 1.7	12.2 ± 2.7	16.0 ± 1.4	17.1 ± 1.8
C20:1n-9 (eicosenoic)	0.3 ± 0.1	0.3 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
C24:1n-9 (nervonic)	0.3 ± 0.2	0.3 ± 0.4	0.6 ± 0.2	0.9 ± 0.5
C18:2n-6 (linoleic)	22.0 ± 3.5[b]	25.1 ± 2.7	13.3 ± 2.8	14.5 ± 1.6
C20:3n-6 (eicosatrienoic)	3.1 ± 0.7	2.8 ± 0.6	1.8 ± 0.4	1.7 ± 0.2
C20:4n-6 (arachidonic)	10.5 ± 1.8[c]	9.4 ± 1.4	14.6 ± 1.3[d]	12.8 ± 1.5
C22:4n-6 (docosatetraenoic)	0.5 ± 0.1	0.5 ± 0.2	3.9 ± 0.9	3.6 ± 0.5
C18:3n-3 (α-linolenic)	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2
C20:5n-3 (eicosapentaenoic)	0.8 ± 0.3	1.0 ± 0.6	0.8 ± 0.2	1.0 ± 0.3
C22:5n-3 (docosapentaenoic)	0.9 ± 0.2	0.9 ± 0.3	2.3 ± 0.6	2.2 ± 0.2
C22:6n-3 (docosaexaenoic)	3.3 ± 0.9	3.4 ± 1.1	4.1 ± 1.1	4.5 ± 1.0

[a] Data are presented as mean ± SD.
[b] $P = 0.028$ *versus* control subjects.
[c] $P = 0.034$ *versus* control subjects.
[d] $P = 0.011$ *versus* control subjects.

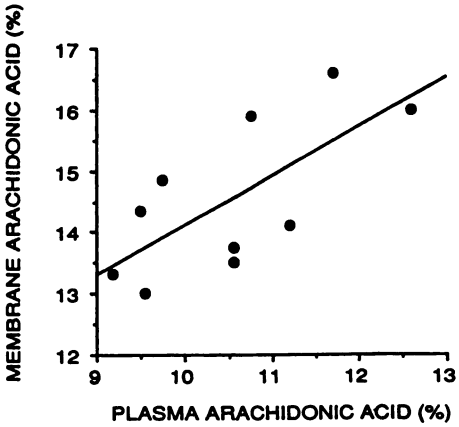

Figure 1. Relationship between plasma and erythrocyte membrane levels of arachidonic acid in phospholipids ($r = 0.75$, $P = 0.033$).

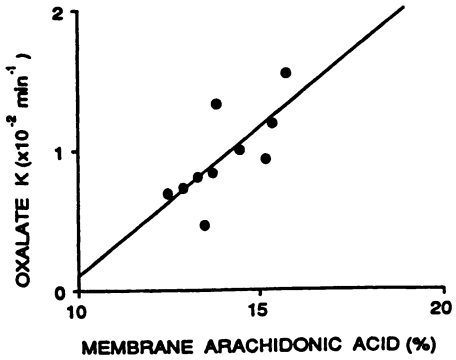

Figure 2. Relationship between erythrocyte membrane levels of arachidonic acid and transmembrane oxalate self-exchange rate (*K*) in stone formers ($r = 0.72$, $P = 0.019$).

levels (Figure 1). The relationship between plasma and erythrocyte membrane composition in phospholipid fatty acids is a complex and still unresolved problem, because of the limited turnover and *de novo* synthesis of phospholipids in RBC. The concomitant increase in arachidonic acid observed in both plasma and RBC membrane phospholipids, and clinical and experimental reports that also show a relationship between plasma phospholipid fatty acids and liver or kidney cell membranes (25,26), suggest that such a change in fatty acid composition is systemic, and possibly involves the kidney.

Regarding the origin of the anomalous arachidonic acid content in plasma and RBC membrane phospholipids, one needs to consider that some long-chain polyunsaturated fatty acids of tissue and serum lipids are obtained in man by desaturation and elongation of their precursors, linoleic acid and α-linolenic acid, which cannot be synthesized by the human body and must be supplied by the diet (13,14). The finding of a higher concentration of arachidonic acid in phospholipids of ICN patients, despite low levels of linoleic acid—the main precursor of arachidonic acid—and the consequent higher arachidonic/linoleic acid ratio suggest that the anomalous arachidonic acid content is not the result of an excessive intake and absorption

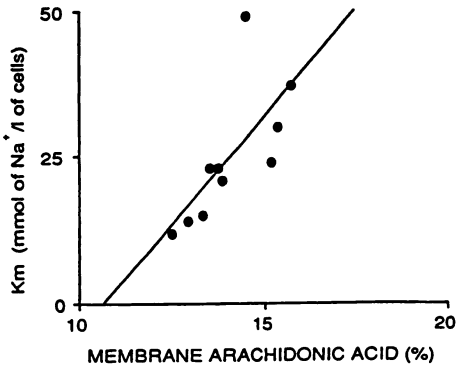

Figure 3. Relationship between erythrocyte membrane levels of arachidonic acid and the Na/K/2Cl cotransport K_m in stone formers (r = 0.70, P = 0.023). Because a higher K_m value indicates a reduced Na/K/2Cl activity, this correlation supports the inhibitory effect of PGE_2 on the sodium cotransporter.

TABLE 4. Plasma lipids before and after n-3 polyunsaturated fatty acid supplementation[a]

Plasma Lipid (mg/dL)	Before	After
Cholesterol	196.2 ± 21.4	206.0 ± 24.4
Triglycerides	94.0 ± 25.6	111.8 ± 27.9
HDL cholesterol	52.5 ± 7.4	49.5 ± 9.7
Phospholipids	202.7 ± 27.3	205.4 ± 27.0

[a] Data are presented as mean ± SD.

TABLE 5. Percentage of fatty acids in the plasma phospholipids before and after n-3 polyunsaturated fatty acid supplementation[a]

Fatty Acid	Before	After	P
C14:0 (myristic)	0.6 ± 0.1	0.8 ± 0.3	
C16:0 (palmitic)	28.4 ± 0.9	31.7 ± 2.6	
C18:0 (stearic)	14.2 ± 0.9	13.9 ± 1.5	
C24:0 (lignoceric)	0.2 ± 0.1	0.3 ± 0.1	
C16:1n-7 (palmitoleic)	0.9 ± 0.2	0.1 ± 0.3	
C18:1n-9 (oleic)	11.9 ± 1.5	11.9 ± 1.9	
C20:1n-9 (eicosenoic)	0.3 ± 0.1	0.3 ± 0.3	
C24:1n-9 (nervonic)	0.2 ± 0.1	0.3 ± 0.1	
C18:2n-6 (linoleic)	24.6 ± 2.7	21.0 ± 1.9	0.017
C20:3n-6 (eicosatrienoic)	3.2 ± 0.1	2.4 ± 0.5	0.016
C20:4n-6 (arachidonic)	10.0 ± 0.8	7.8 ± 1.3	0.002
C22:4n-6 (docosatetraenoic)	0.5 ± 0.1	0.5 ± 0.2	
C18:3n-3 (α-linolenic)	0.2 ± 0.1	0.4 ± 0.1	
C20:5n-3 (eicosapentaenoic)	0.7 ± 0.2	2.6 ± 1.4	0.022
C22:5n-3 (docosapentaenoic)	0.9 ± 0.2	1.2 ± 0.5	
C22:6n-3 (docosaexaenoic)	3.5 ± 1.0	5.8 ± 1.0	0.018

of the parent dietary fatty acids, but is the consequence of a primary derangement in arachidonic acid synthesis, perhaps caused by an increased activity of desaturases, the rate-limiting step of the biosynthetic pathway of highly unsaturated fatty acids (27).

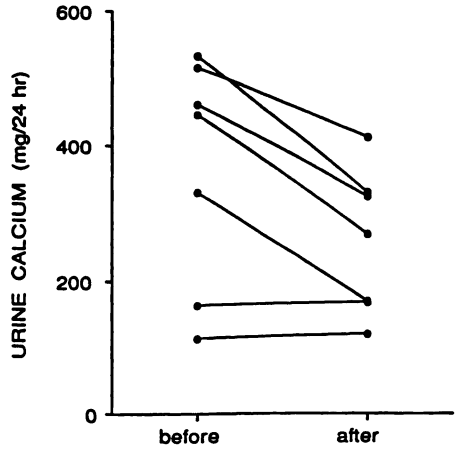

Figure 4. Effect of fish-oil diet supplementation on urinary calcium (t = 3.07, P = 0.022).

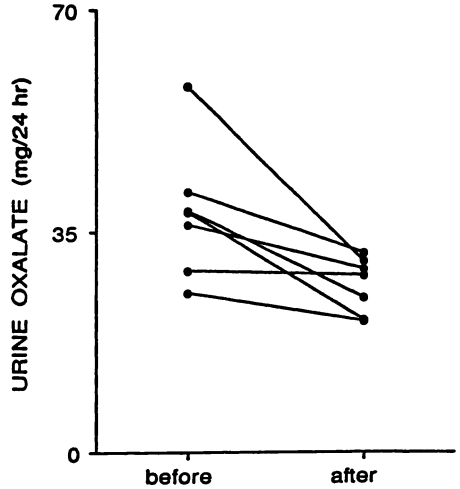

Figure 5. Effect of fish-oil diet supplementation on urinary oxalate (t = 3.39, P = 0.015).

Whatever the mechanism is that underlies the rise in the phospholipid arachidonic acid proportion of RBC membranes, the abnormal phospholipid fatty acid composition might in itself be responsible for the mosaic of ion transport anomalies observed in ICN, and (because of the systemic extension of the phospholipid fatty acid anomaly) possibly the secondary urinary abnormalities (hyperprostaglandinuria, hypercalciuria, hyperoxaluria, *etc.*) as well.

Indeed, an arachidonic acid increase in the membrane phospholipids is known to result in an elevation in PGE_2 production. Consistent with this are the increased PGE_2 urinary levels observed in these patients and data from the literature in hypercalciuric stone formers, which suggest that hyperprostaglandinuria is distinctive of idiopathic nephrolithiasis (10–12,18). PGE_2 may cause hypercalciuria by facilitating intestinal calcium absorption because of its

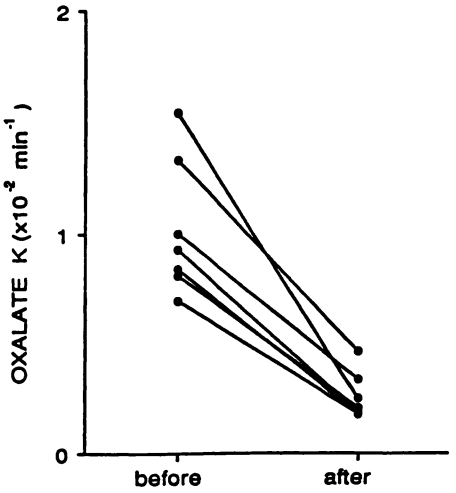

Figure 6. Effect of fish-oil diet supplementation on the erythrocyte oxalate self-exchange (K) (t = 7.85, P = 0.000).

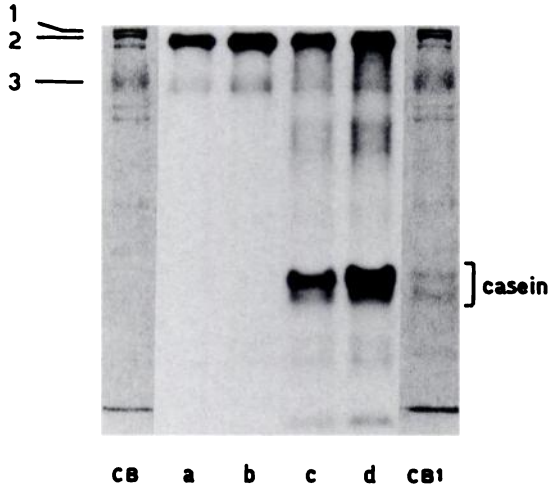

Figure 7. Effect of phospholipase A_2 on RBC membrane protein phosphorylation. When added to RBC, phospholipase A_2 increased ^{32}P incorporation in band 2 and 3 proteins (40 and 25%, respectively) (Lane b *versus* Lane a, control), and the casein kinase activity (about 50%) (Lane d *versus* Lane c, control). Lanes CB and CB1 show the Coomassie blue-stained gels of membrane proteins and of casein, respectively. The figure shows results from a single subject. Similar data were obtained in four other healthy control subjects.

stimulation of 1α-hydroxylase and consequently 1,25-dihydroxyvitamin D synthesis (28). Moreover, PGE_2 could induce hypercalciuria by its inhibitory effect on the Na/K/2Cl co-transporter (29–31), the activity of which we found really to be decreased (*i.e.*, a higher K_m) in these patients, both in erythrocytes and kidney (5), and correlated with arachidonic acid levels in RBC membrane phospholipids (Figure 3). Inhibited Na/K/2Cl cotransport activity in the thick ascending limb of Henle's loop reduces the transepithelial potential difference, and thus decreases the paracellular tubular reabsorption of calcium, leading to hypercalciuria (5).

The link between the anomalous arachidonic acid level in membrane phospholipids and urine oxalate excretion is more complex. A direct relationship between erythrocyte membrane levels of arachidonic acid and transmembrane oxalate self-exchange rate has been observed (Figure 2). This cell anomaly, the most-studied cell defect among the many observed in ICN (1,2,32), seems to be genetically transmitted, is found in about 70% of ICN patients, and is associated with increased oxalate renal clearance (33) and renal stone activity (34). It depends on biochemical alterations in the anion carrier, the so-called band 3 protein, which, together with band 2 protein, is more phosphorylated in stone formers (35), probably because of an increased activity of the cAMP and Ca^{2+} independent Ser/Thr-protein kinases (casein kinases CK1 and CK2) (36,37). In this setting, considering the important role of membrane phospholipids in regulating membrane-mediated phenomena, or in modulating protein kinases (7–9), we may speculate that changes in membrane phospholipid fatty acid pattern, by modulating protein kinases responsible for band 2 and 3 protein phosphorylation, induce abnormal RBC and renal oxalate transport.

The observation that phospholipase A_2, the key enzyme in arachidonic acid release from membrane phospholipids, increases band 2 and band 3 protein phosphorylation through the activation of casein kinases (Figure 7), and concomitantly augments band 3 exchanging function, as documented by the increase in RBC oxalate transport, strongly supports the proposed mechanism.

The hypothesis of a link between arachidonic acid level in membrane phospholipids and cellular and urinary anomalies observed in ICN seems to be confirmed by the hypocalciuric and hypooxaluric effect of fish-oil dietary supplementation (Figures 4 and 5). These data, although obtained in few patients, are in agreement with well-known literature reports; in fact, Buck *et al.* (15) demonstrated a reduction in urine calcium and oxalate excretion with fish-oil treatment over a 8-wk period. It is also known that dietary manipulation with fish oil determines a shift from the n-6 to the n-3 polyunsaturated fatty acid series (13,14), and specifically leads, as confirmed by this study, to a decrease in the linoleic and arachidonic acid levels, and in an increase in the proportions of 20:5(n-3) and 22:6(n-3) polyunsaturated fatty acids in phospholipids (Tables 4 and 5). In view of literature data demonstrating contemporaneous effects of fish-oil supplements on membrane phospholipids of RBC, liver, and kidney cells (26,38,39), it seems likely that the changes we observed in plasma occurred in RBC membranes as well. Hence, we advance the hypothesis that dietary polyunsaturated fatty acids by affecting membrane phospholipid fatty acid composition modify protein kinase activities, change band 3 phos-

phorylation level, and consequently normalize the oxalate cellular self-exchange (Figure 6); this cellular mechanism could explain the reduction of urinary oxalate excretion (Figure 5) reported by Buck *et al.* as well (15).

Furthermore, dietary n-3 polyunsaturated fatty acids, attenuating tissue arachidonic acid levels, modulate systemic eicosanoid production and, in particular, induce a significant reduction in PGE_2 synthesis in the kidney, as well as its urinary excretion (13,26,40). This PGE_2 decrease might lead to a disinhibition/activation in renal Na/K/2Cl cotransport function, and thus determine a higher renal tubular reabsorption of sodium and calcium, and subsequently a lower calcium excretion. On the other hand, many workers report that dietary and experimental manipulations that are able to modify membrane phospholipid fatty acids, influence the Na/K/2Cl cotransport activity and other ion transports by modulating cell signal transduction (41–43).

In conclusion, this study shows that our ICN patients have a systemic defect in their phospholipid arachidonic acid level that may be significant in determining the anomalous activity of a number of cell ion carriers in different tissues, and in inducing hypercalciuria and hyperoxaluria, well-known risk factors for lithogenesis. Moreover, it foresees a link between genetic background, environmental factors (dietary habits), and nephrolithiasis. In fact, the activity of the fatty acid 6-desaturase, the key enzyme in the arachidonic acid synthesis, is modulated by nutritional factors, and specifically by protein and glucose intakes, and by the dietary lipid composition (27). So, a particular genetic trait of the desaturase enzyme, or an acquired dietary-induced defect of it, or both mechanisms together might induce an abnormal arachidonic acid metabolism, thus playing a role in the pathogenesis of nephrolithiasis. In this regard, it is worth noting that Eskimos, who appear to lack desaturases (44) and consume a n-3 fatty acid-rich diet, do not generally develop nephrolithiasis (17), and finally, that idiopathic calcium nephrolithiasis, from an epidemiologic point of view, is a disease that appeared in Western countries at the beginning of this century, closely correlated with changing dietary habits (predominant ingestion of n-6 fatty acids and proteins) and economic affluency.

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REFERENCES

1. **Baggio B, Gambaro G, Marchini F, Cicerello E, Borsatti A:** Raised transmembrane oxalate flux in red blood cells in idiopathic calcium oxalate nephrolithiasis. Lancet 1984;2:12–13.
2. **Baggio B, Gambaro G, Marchini F, *et al.*:** An inheritable anomaly of red-cell oxalate transport in "primary" calcium nephrolithiasis correctable with diuretics. N Engl J Med 1986;314:599–604.
3. **Gambaro G, Vincenti M, Marchini F, D'Angelo A, Baggio B:** Abnormal urate transport in erythrocytes of idiopathic calcium nephrolithiasis: A possible link with hyperuricosuria. Clin Sci (Lond) 1993;85:41–44.
4. **Bianchi G, Vezzoli G, Cusi D, *et al.*:** Abnormal red-cell calcium pump in patients with idiopathic hypercalciuria. N Engl J Med 1988;319:897–901.
5. **Baggio B, Gambaro G, Marchini F, *et al.*:** Abnormal erythrocyte and renal frusemide-sensitive sodium transport in idiopathic calcium nephrolithiasis. Clin Sci (Lond) 1994;86:239–243.
6. **Baggio B, Gambaro G, Marchini F, Marzaro G, Williams HE, Borsatti A:** Correction of erythrocyte abnormalities in idiopathic calcium oxalate nephrolithiasis and reduction of urinary oxalate by oral glycosaminoglycans. Lancet 1991;338:403–405.
7. **Pelech SL, Vance DE:** Signal transduction via phosphatidylcholine cycles. TIBS (Trends Biochem Sci) 1989; 14:28–30.
8. **Liscovitch M, Cantley LC:** Lipid second messengers. Cell 1994;77:329–334.
9. **Exton JH:** Phosphatidylcholine breakdown and signal transduction. Biochim Biophys Acta 1994;1212:26–42.
10. **Buck AC, Lote CJ, Sampson WF:** The influence of renal prostaglandins on urinary calcium excretion in idiopathic urolithiasis. J Urol 1983;129:421–426.
11. **Hirayama H, Ikegami K, Shimomura T, Soejima H, Yamamoto T:** The possible role of prostaglandins E2 in urinary stone formation. J Urol 1988;139:549–551.
12. **Henriquez-La Roche C, Rodriguez-Iturbe B, Parra G:** Increased urinary excretion of prostaglandin E2 in patients with idiopathic hypercalciuria is a primary phenomenon. Clin Sci (Lond) 1992;83:75–80.
13. **Fischer S:** Dietary polyunsaturated fatty acids and eicosanoid formation in humans. Adv Lip Res 1989;23:169–198.
14. **Davidson J:** Lipid metabolism. Curr Opin Lipidol 1995; 6:U65–U66.
15. **Buck AC, Davies RL, Harrison T:** The protective role of eicosapentaenoic acid (EPA) in the pathogenesis of nephrolithiasis. J Urol 1991;146:188–194.
16. **Rothwell PJN, Green R, Blacklock NJ, Kavanagh JP:** Does fish oil benefit stone formers? J Urol 1993;150: 1391–1394.
17. **Modlin M.** Urinary sodium and renal stones. In: Hodgkinson A, Nordin BEC, Eds. Renal Stone Symposium. London: Churchill; 1969:209–220.
18. **Gambaro G, Marchini F, Vincenti M, *et al.*:** Is hyperprostaglandinuria a primitive phenomenon in idiopathic calcium nephrolithiasis? [Abstract.] J Am Soc Nephrol 1994;5:862A.
19. **Folch J, Lees M, Stanley GHS:** A single method for the isolation and purification of total lipid from animal tissue. J Biol Chem 1957;226:497–507.
20. **Rose HG, Oklander M:** Improved procedure for the extraction of lipids from human erythrocytes. J Lipid Res 1965;6:428–431.
21. **Morrison WR, Smith LM:** Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J Lipid Res 1964;5:600–608.
22. **Bartlett GR:** Phosphorus assay in column chromatography. J Biol Chem 1959;234:466–468.
23. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ:** Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:256–275.
24. **Bordin L, Clari G, Bellato M, Tessarin C, Moret V:** Effect of okadaic acid on membrane protein phosphorylation in human erythrocytes. Biochem Biophys Res Commun 1993;195:723–729.
25. **Engelmann B, Streich S, Schonthier UM, Richter WO, Duhm J:** Changes of membrane phospholipid composition of human erythrocytes in hyperlipidemias. I.Increased phosphatidylcholine and reduced sphingomye-

lin in patients with elevated levels of triacylglycerol-rich lipoproteins. Biochim Biophys Acta 1992;1165:32–37.
26. **Kawashima Y, Mizuguchi H, Kozuka H:** Modulation by dietary oils and clofibric acid of arachidonic acid content in phosphatidylcholine in liver and kidney of rat. Effects on prostaglandin formation in kidney. Biochim Biophys Acta 1994;1210:187–194.
27. **Brenner RR.** Factors influencing fatty acid chain elongation and desaturation. In: Vergroesen AJ, Crawford M, Eds. The Role of Fats in Human Nutrition. 2nd Ed. London: Academic Press; 1989:45–79.
28. **Kurokawa K:** Calcium-regulating hormones and the kidney. Kidney Int 1987;32:760–771.
29. **Stokes JB:** Integrated actions of renal medullary prostaglandins in the control of water excretion. Am J Physiol 1981;240:F471–F480.
30. **Molony DA, Reeves WB, Andreoli TE:** Na:K:2Cl cotransport and the thick ascending limb. Kidney Int 1989;36: 418–426.
31. **Kaji D:** Prostaglandin E2 (PGE2) inhibits NaK2Cl cotransport in cultured mouse medullary thick ascending limb [Abstract.] J Am Soc Nephrol 1991;2:428A..
32. **Motola JA, Urivetsky M, Molia L, Smith AD:** Transmembrane oxalate exchange: Its relationship to idiopathic calcium oxalate nephrolithiasis. J Urol 1992;147:549–552.
33. **Gambaro G, Petrarulo M, Nardelotto A, Marangella M, Baggio B:** Erythrocyte transmembrane flux and renal clearance of oxalate in idiopathic calcium nephrolithiasis. Kidney Int 1995;48:1549–1552.
34. **Gambaro G, Marchini F, Vincenti M, Budakovic A, Nardellotto A, Baggio B:** Clinical features of idiopathic calcium-oxalate nephrolithiasis associated with the anomalous erythrocyte self-exchange of oxalate [Abstract.] Proceedings of the 5th European Urolithiasis Symposium, Manchester, UK, 21–23 April, 1994:67A.
35. **Baggio B, Clari G, Marzaro G, Gambaro G, Borsatti A, Moret V:** Altered red blood cell membrane protein phosphorylation in idiopathic calcium oxalate nephrolithiasis. IRCS (Int Res Commun Syst) Med Sci 1986;14:368–369.
36. **Baggio B, Bordin L, Gambaro G, Piccoli A, Marzaro G, Clari G:** Evidence of a link between erythrocyte band 3 phosphorylation and anion transport in patients with "idiopathic" calcium oxalate nephrolithiasis. Miner Electrolyte Metab 1993;19:17–20.
37. **Baggio B, Bordin L, Clari G, Gambaro G, Moret V:** Functional correlation between the Ser/Thr-phosphorylation of band-3 and band-3 mediated transmembrane anion transport in human erythrocytes. Biochim Biophys Acta 1993;1148:157–160.
38. **Popp-Snijders C:** Assessment of compliance to changes in dietary fatty acids. Diab Nutr Metab 1991;4:155–163.
39. **Marangoni F, Angeli MT, Colli S, *et al.*:** Changes of n-3 and n-6 fatty acids in plasma and circulating cells of normal subjects, after prolonged administration of 20:5 (EPA) and 22:6 (DHA) ethyl esters and prolonged washout. Biochim Biophys Acta 1994;1210:55–62.
40. **Düsing R, Struck A, Gobel BO, Weisser B, Vetter H:** Effects of n-3 fatty acids on renal function and renal prostaglandin E metabolism. Kidney Int 1990;38:315–319.
41. **Pagnan A, Corrocher R, Ambrosio GB, *et al.*:** Effects of olive-oil-rich diet on erythrocyte membrane lipid composition and cation transport systems. Clin Sci (Lond) 1989;76:87–93.
42. **Engelmann B, Op Den Kamp JAF, Roelofsen B:** Replacement of molecular species of phosphatidylcholine: Influence on erythrocyte Na transport. Am J Physiol 1990; 258:C682–C691.
43. **Dwight JFStJ, Mendes Ribeiro AC, Hendry BM:** Membrane incorporation of non-esterified fatty acids and effects on the sodium pump of human erythrocytes. Clin Sci (Lond) 1992;82:99–104.
44. **Gibson RA, Sinclair AJ:** Are eskimos obligated carnivores? Lancet 1981;1:1100–1101.