Calcium-phosphate metabolism parameters and erythrocyte Ca^{2+} concentration in autosomal dominant polycystic kidney disease patients with normal renal function

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Abstract

Introduction: The aim of this study was to assess calcium-phosphate metabolism of autosomal dominant polycystic kidney disease (ADPKD) patients with a special consideration to the following serum parameters: calcium (Ca^{2+}), inorganic phosphate (Pi), parathyroid hormone (PTH) and intracellular erythrocyte calcium ($[Ca^{2+}]_i$) concentrations.

Material and methods: The study included 49 adult ADPKD patients (19 males, 30 females) aged 36 ±11 years with normal renal function and no diagnosis of diabetes as well as 50 healthy controls (22 males, 28 females) matched for age and gender. Serum concentrations of sodium (Na^+), potassium (K^+) and magnesium (Mg^{2+}) ions and Pi were determined with an indirect ion-selective method, while Ca^{2+} concentration was measured with a direct ion-selective method. The PTH was detected using a radioimmunometric method. $[Ca^{2+}]_i$ concentration was determined with the Ca^{2+} sensitive fluorescent dye Fura-2 method.

Results: In the ADPKD group, when compared to controls, the following concentrations were significantly higher: serum Ca^{2+} (1.18 ±0.06 mmol/l vs. 1.15 ±0.06 mmol/l, $p = 0.0085$), $[Ca^{2+}]_i$ (146.9 ±110.0 nmol/l vs. 96.5 ±52.7 nmol/l, $p = 0.0075$), serum Na^+ (139.4 ±2.7 mmol/l vs. 138.5 ±2.1 mmol/l, $p = 0.060$, borderline significance), and PTH (15.5 ±6.8 pg/ml vs. 13.6 ±5.3 pg/ml, $p = 0.066$, borderline significance), while serum Mg^{2+} was significantly lower (0.81 ±0.09 mmol/l vs. 0.85 ±0.05 mmol/l, $p = 0.021$). In the ADPKD group we observed significant negative correlations of PTH with Ca^{2+} serum concentrations ($Rs = -0.32$, $p = 0.025$) and with estimated glomerular filtration rate ($Rs = -0.31$, $p = 0.033$).

Conclusions: The erythrocyte Ca^{2+} concentration is elevated in ADPKD patients with normal renal function. It may result from a dysfunction of mutated polycystins which can affect various aspects of electrolyte metabolism.

Key words: calcium, magnesium, inorganic phosphate, parathormone, polycystins.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary kidney disease, with a prevalence of 1 : 400 to 1 : 1000 in Caucasians. In Europe approximately 6% of all patients with chronic renal replacement therapy are kidney insufficient due to ADPKD [1]. The ADPKD results from mutations in the *PKD1* gene (in about 85% of cases) located on chromosome 16 [2] as well as in the *PKD2* gene on chromosome 4 [3]. These genes encode respectively polycystin-1 (PC-1) and polycystin-2 (PC-2) proteins [4], which work in a common cellular pathway. The PC-1 is a large receptor molecule forming a receptor-channel complex with PC-2, which is a cation channel from the transient receptor potential (TRP) family [5]. PC-1 and PC-2 proteins assemble in the plasma membrane to regulate the calcium (Ca^{2+}) entry mechanism [6]. It is thought that renal epithelial cell hyperplasia in ADPKD patients is a consequence of dysfunctional Ca^{2+} metabolism following polycystin protein mutations [7].

Specific roles of PC-1 and PC-2 in intracellular calcium ($[Ca^{2+}]_i$) regulation as well as the pathway of epithelial cell hyperplasia and cyst formation due to *PKD* gene mutations still remain unclear.

Yamaguchi *et al*. noted that a reduction of $[Ca^{2+}]_i$ in renal cyst epithelial cells due to mutations in *PKD* genes releases protein kinase B (Akt) inhibition of serine/threonine-protein kinase B-Raf, which promotes cyclic adenosine monophosphate (cAMP)-dependent cell proliferation and cyst growth. They have found that an increase of $[Ca^{2+}]_i$ in polycystic kidney cells can lead to enhanced Akt activity which represses cAMP-dependent stimulation of B-Raf as well as extracellular signal-regulated kinases (ERKs) and cell proliferation and thus restore a normal antimitogenic response to cAMP.

Sustained reduction of $[Ca^{2+}]_i$ with L-type calcium channel blockers (verapamil and nifedipine) predisposes cells derived from normal human kidney to cAMP-dependent activation of the B-Raf/MEK/extracellular signal regulated kinase (B-Raf/MEK/ERK) pathway and leads to increased cell proliferation, which mimics the ADPKD phenotype. Treatment of ADPKD cells with calcium channel blockers (CCB) amplifies cAMP-dependent ERK activation and proliferation, which suggests that further reduction in $[Ca^{2+}]_i$ may accelerate cyst growth [8].

Calcium-phosphate metabolism disturbances develop in chronic kidney disease patients during early stages of renal failure [9], but little is known about metabolic disturbances in ADPKD patients before the onset of renal failure.

The aim of this study was to assess calcium-phosphate metabolism of ADPKD patients with normal renal function with a special consideration to serum concentrations of calcium (Ca^{2+}), inorganic phosphate (Pi), parathyroid hormone (PTH), as well as erythrocyte calcium concentration ($[Ca^{2+}]_i$).

Material and methods

The study group initially included 50 adult individuals with ADPKD diagnosis (20 males, 30 females), while the control group comprised 50 gender- and age-matched healthy individuals (22 males, 28 females).

For the study group the following inclusion criteria were applied: the presence of cysts in both kidneys according to the Ravine *et al*. criteria of the PKD phenotype [10], ADPKD in family history, serum creatinine concentration ≤ 120 µmol/l, and a negative history of diabetes. One patient with serum creatinine elevated to 162 µmol/l at the time of examination was excluded from the study. The final study group consisted of 49 subjects (19 males, 30 females). Individuals with a negative family history of ADPKD, an absence of cysts in kidneys (Ravine's criteria not fulfilled), serum creatinine concentration ≤ 120 µmol/l, and no prior diagnosis of diabetes, were enrolled for the control group.

Each participant was thoroughly informed about the study and asked for written consent to participate. The study protocol was approved by the Ethical Committee of the Pomeranian Medical University, Szczecin, Poland (approval No. 001/135/06).

At the baseline a full medical history review and a clinical examination was obtained from each participant. Blood pressure was measured twice at 2-minute intervals after a 10-minute rest in the sitting position and the mean value was used in analyses. Hypertension was defined as systolic/diastolic blood pressure ≥ 140/90 mm Hg or treatment with antihypertensive drugs.

The serum concentrations of Na^+, K^+, Mg^{2+} ions and Pi were determined with an indirect ion-selective method using the Cobas Integra 800 bioanalyzer (Roche, reagents of Roche company). Ca^{2+} concentrations in serum were estimated with a direct ion-selective method using the CIBA-Corning 634 analyzer (Bayer). Serum creatinine concentrations were measured with the Cobas Integra 800 bioanalyzer (Roche).

The estimated glomerular filtration rate (eGFR) was calculated according to the Modification of Diet in Renal Disease (MDRD) simplified formula on the basis of a single serum creatinine measurement [11].

Determination of intracellular free Ca^{2+} ion concentrations in human erythrocytes

Anticoagulant (2.73% citric acid, 4.48% sodium citrate, 2% glucose) collected blood was centrifuged (20°C, 5 min, 750 g) and plasma with leukocyte buffy coat was removed. Erythrocytes were diluted with HBS buffer (123 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$,

1 mM $CaCl_2$, 10 mM glucose, 25 mM HEPES, pH = 7.4) to 1% hematocrit and incubated with 1 µM solution of Fura-2-acetoxymethyl ester (Fura-2AM; Sigma) dissolved in DMSO for 45 min at 37°C. After the addition of Fura-2AM, all activities were performed in the darkness. After incubation, erythrocytes were rinsed with HBS buffer to remove excess Fura-2AM and diluted with the same buffer to 0.02% hematocrit. Next the fluorescence was measured using the Perkin Elmer LS 50 B spectrometer at excitation wavelengths of 340 nm and 380 nm and a constant emission wavelength of 510 nm. The fluorescence of erythrocytes without Fura-2AM was also measured to compensate for erythrocyte endogenous fluorescence. As calibration, the fluorescence of Fura-2AM incubated erythrocytes with the addition of 4% Triton X-100 and 10 mM EGTA was measured at the same wavelengths according to the procedure described by Soldati *et al*. [12].

Determination of the parathyroid hormone (PTH) concentration

PTH concentration was determined with a radioimmunometric method by means of ^{125}I labeled monoclonal antibodies specific for the 44-68 hPTH fragment. Measurements were performed using the BioSource hPTH-120 min-IRMA kit (BioSource Europe SA, Nivelles, Belgium, catalog no. KIP, 1491) according to the manufacturer's directions. Radioactivity was measured in a gamma scintillation counter for more than 60 s and results were calculated by the RIA-CALC software package on the basis of calibration curves.

Statistical analysis

Since quantitative variables did not have normal distribution, the Mann-Whitney test was used. For qualitative variables the Fisher exact test was applied. The Spearman rank correlation coefficient (*Rs*) was used to measure associations between quantitative variables. Differences with $p < 0.05$ were considered as statistically significant. The data are presented as a number (percentage) for qualitative variables or as a mean value ± standard deviation for quantitative variables. Statistica 7.1 software was used for all statistical analyses.

Results

Anthropometric, biochemical parameters and pharmacological treatment of hypertension (HT) of ADPKD patients and control groups are presented in Table I. Hypertension was more commonly diagnosed in the study group than among controls. The ADPKD patients were more often treated with angiotensin-converting enzyme inhibitors (ACE inhibitors) and thiazide-like diuretics (indapamide in all cases). No participant received CCB. Parameters of calcium-phosphate metabolism and concentrations of other ions are presented in Table II. The ADPKD patients showed a significantly higher Ca^{2+} concentration, a significantly lower Mg^{2+} concentration, borderline higher concentrations of Na^+ and PTH in serum, as well as a significantly higher Ca^{2+} concentration in erythrocytes. There were no significant differences in serum concentrations of K^+ and Pi.

The presence of HT in ADPKD patients was associated with a significantly lower concentration of Pi (0.98 ±0.15 mmol/l for HT patients vs. 1.08 ±0.17 mmol/l for patients without HT, $p = 0.031$), but no significant associations with other ion concentrations were found.

Studied parameters were also compared in patients without HT (20 ADPKD patients and 46 controls). A comparison of the non-HT ADPKD subgroup with the non-HT control subgroup showed, similarly as in the entire group, significantly higher $[Ca^{2+}]_i$ con-

Table I. Clinical characteristics of the ADPKD patients and the control group

Parameters	ADPKD group ($n = 49$)	Control group ($n = 50$)	Value of p^a
Age [years]	35.9 ±11.1	36.7 ±9.2	0.62
Sex (% males)	19 (39%)	22 (44%)	0.68
BMI [kg/m^2]	25.1 ±4.9	24.4 ±3.7	0.63
Creatinine [mg/dl]	0.84 ±0.18	0.80 ±0.15	0.54
eGFR [ml/min/1.73 m^2]	98.0 ±21.1	103.1 ±19.9	0.27
Hypertension	29 (59%)	4 (8%)	< 0.00001
ACE inhibitors	27 (55%)	3 (6%)	< 0.001
β-Blockers	3 (6%)	0 (0%)	0.12
Diuretics (indapamide)	12 (24%)	3 (6%)	0.012

Data are given as mean ± SD or number (percentage) of patients. ACE inhibitors – angiotensin-converting enzyme inhibitors, BMI – body mass index, eGFR – estimated glomerular filtration rate. [a]ADPKD vs. control group; Fisher exact test for qualitative variables and Mann-Whitney test for quantitative variables were used

Table II. Comparison of the ion and parathormone serum concentrations, and erythrocyte calcium concentrations in the ADPKD patients and the control group

Parameter	ADPKD group (n = 49)	Control group (n = 50)	Value of p[a]
Na^+ [mmol/l]	139.4 ±2.7	138.5 ±2.1	0.060
K^+ [mmol/l]	4.22 ±0.40	4.18 ±0.35	0.96
Ca^{2+} [mmol/l]	1.18 ±0.06	1.15 ±0.06	0.0085
Mg^{2+} [mmol/l]	0.81 ±0.09	0.85 ±0.05	0.021
Pi [mmol/l]	1.02 ±0.17	1.06 ±0.14	0.20
PTH [pg/ml]	15.5 ±6.8	13.6 ±5.3	0.066
Erythrocyte calcium [nmol]	146.9 ±110.0	96.5 ±52.7	0.0075

[a]*ADPKD vs. control group; Mann-Whitney test. Pi – inorganic phosphate, PTH – parathormone*

centration (175.9 ±96.9 nmol/l vs. 150.8 ±51.3 nmol/l, p = 0.022), significantly lower serum Mg^{2+} concentration (0.80 ±0.08 mmol/l vs. 0.85 ±0.05 mmol/l, p = 0.013) and higher serum Na^+ concentration (139.4 ±2.2 mmol/l vs. 138.5 ±2.2 mmol/l, p = 0.060, borderline significance). However, the difference in serum Ca^{2+} concentrations was not significant (p = 0.22). Treatment with any drugs of the three antihypertensive groups was not associated with significant differences in studied ion concentrations.

In the ADPKD group we also observed significant negative correlations of PTH with serum Ca^{2+} concentration (Rs = –0.32, p = 0.025) and with eGFR (Rs = –0.31, p = 0.033). There were no significant correlations between serum PTH and other ion concentrations (Na^+, K^+, Mg^{2+}, Pi). $[Ca^{2+}]_i$ concentration was also not correlated with concentrations of analyzed ions in serum.

Discussion

We found that ADPKD patients with normal renal function showed higher Ca^{2+} concentrations both in serum and in erythrocytes, lower Mg^{2+} serum concentration, and higher serum PTH levels (borderline significance), than individuals in the control group.

Most ADPKD patients have hypertension before the onset of renal failure [13]. Arterial hypertension treatment may lead to various electrolyte disorders: ACE inhibitors may lead to hyperkalemia, CCB to a reduction of Ca^{2+} in erythrocytes [14], and thiazide diuretics to hypomagnesemia and hypocalcemia [15]. In our study we did not observe correlations between ion concentrations and administration of antihypertensive drugs. It should be noted, however, that nobody was treated with CCB or thiazide diuretics (patients received indapamide, which does not induce hypocalcemia).

We also observed that PTH levels were higher in patients with lower concentrations of eGFR and Ca^{2+}. The only study concerning correlation of eGFR with PTH in ADPKD patients in early stages of renal failure was performed by Fliser *et al*. [16], who observed that in a group of ADPKD and IgA glomerulonephritis patients a deterioration in renal function (creatinine concentration groups < 1.3, 1.3-3.0, > 3.0 mg/dl) was accompanied by a significant increase in PTH levels (4.7 ±0.4 pmol/l, 8.4 ±1.6 pmol/l, 39.6 ±7.9 pmol/l respectively). In our study the correlation between PTH levels and eGFR was also negative.

Studies on patients with chronic kidney disease have shown that an increase in PTH secretion develops in early stages of renal failure (ERF) [17-19] and it is negatively correlated with serum Ca^{2+} concentrations [19]. Similarly, our ADPKD patients with normal renal function showed higher than healthy controls PTH serum levels (borderline significance), which were also negatively correlated with serum Ca^{2+} concentrations. However, our ADPKD patients showed elevated Ca^{2+} serum concentrations, which was not observed in ERF patients.

It seems that higher Ca^{2+} serum levels might be induced by elevated PTH levels. These might also be responsible for the increased Ca^{2+} content in erythrocytes observed in our study. According to Paraskevopoulos *et al*. an enhanced passive Ca^{2+} uptake by erythrocytes observed in uremic patients may be induced by hyperparathyroidism [20]. However, in our study intracellular erythrocyte Ca^{2+} concentrations did not correlate with PTH or other analyzed ion concentrations in serum. Buemi *et al*. reported an anomaly in K^+/Ca^{2+} induced transport in erythrocytes of subjects with ADPKD and hypertension [21]. Factors leading to an elevation of erythrocyte $[Ca^{2+}]_i$ concentration in ADPKD patients with normal renal function need further research.

We have not found studies on erythrocyte Ca^{2+} concentration in ADPKD patients with ERF and there are only a few studies on intracellular calcium content in other ERF patients. Lajdova *et al*. [22] discovered a significantly higher concentration of Ca^{2+} in peripheral blood mononuclear cells in early stages (2-3) of chronic kidney disease (median 123 nmol/l vs. 102 nmol/l, p < 0.001) when compared to a control group. Soldati *et al*. [23] observed, similarly to other

studies on patients with advanced renal failure [20], a significantly higher Ca^{2+} content inside erythrocytes of hemodialyzed patients than in the control group (mean: 101 nmol/l vs. 85 nmol/l, $p < 0.001$). In our study the difference in erythrocyte Ca^{2+} concentrations between the ADPKD and control groups was even higher (mean: 146.9 nmol/l vs. 95.5 nmol/l, $p = 0.0075$).

Only one study has concerned $[Ca^{2+}]_i$ concentration inside kidney cells of ADPKD patients [7]. Yamaguchi *et al*. demonstrated *in vitro* that Ca^{2+} concentration in primary epithelial cell cultures prepared from multiple superficial cysts obtained from kidneys of ADPKD patients is lower than in cells from cortex of normal human kidneys (NHK) (mean: 76.5 nmol/l vs. 56 nmol/l, respectively). The authors also tested cells from cystic and non-cystic regions of early stage ADPKD kidneys removed from patients with relatively normal renal function. They found that Ca^{2+} content in cystic cells was 21.9 nmol/l lower than in non-cystic cells (mean: 40.6 nmol/l vs. 62.5 nmol/l, respectively). Based on these results, Yamaguchi *et al*. suggested that a higher $[Ca^{2+}]_i$ concentration in non-cystic cells of ADPKD patients plays a protective role against development of cysts. It provides an anti-mitogenic response to cAMP, which plays a central role in cystogenesis by stimulating both transepithelial fluid secretion and cyst epithelial cell proliferation [24]. *In vitro* studies have demonstrated that cAMP agonists such as arginine vasopressin (AVP) promote proliferation of epithelial cells derived from ADPKD patients [25]. In contrast, cAMP agonists inhibit proliferation of cells from NHK. The molecular mechanism of phenotypic differences in the cAMP mitogenic response between NHK and ADPKD cells is linked to cAMP-dependent B-Raf signaling to MEK, a kinase that stimulates extracellular signal-regulated kinases (ERKs). In ADPKD cells cAMP activates B-Raf to stimulate the MEK/ERK pathway and cell proliferation, while in NHK B-Raf is inhibited by Akt [26].

Results presented in the Yamaguchi *et al*. study [7] provide evidence that $[Ca^{2+}]_i$ is the central regulator of the mitogenic response to cAMP in human renal epithelial cells. In non-cystic ADPKD cells cAMP decreases ERK activity and inhibits cell proliferation. In contrast, in ADPKD cyst-derived cells, cAMP stimulates ERK and cell proliferation. Thus cyst-derived cells, which presumably have both germline and somatic mutations in the *PKD* genes, are characterized by a lower $[Ca^{2+}]_i$ and cAMP-dependent proliferative phenotype, whereas non-cystic cells from ADPKD kidneys have a normal $[Ca^{2+}]_i$ concentration and a normal antiproliferative response to cAMP [7]. According to our study, Ca^{2+} concentration in erythrocytes of ADPKD patients is higher than in erythrocytes of matched healthy individuals, while according to Yamaguchi Ca^{2+} concentration in renal cells of ADPKD patients is lower than in healthy individuals. Thus Ca^{2+} metabolism differs in different types of human cells and erythrocytes certainly cannot serve as a model of $[Ca^{2+}]_i$ disorders in kidney cells in these patients.

Damage of tubules leads to their dysfunction and different electrolyte disorders. The lower serum Mg^{2+} concentration observed in our ADPKD patients might hypothetically be due to a secondary Fanconi syndrome, which can occur in the course of polycystic kidney disease [27]. This defect of the proximal tubule affects reabsorption of amino acids, glucose, phosphates, sometimes also bicarbonates, uric acid, citrate, low-molecular-weight proteins and some ions: Mg^{2+}, Ca^{2+} and K^+. However, the significantly higher Ca^{2+} serum concentration and the lack of differences in serum phosphate levels observed in our study is not consistent with symptoms of Fanconi syndrome.

In conclusion, an elevated PTH level and its negative correlations with serum Ca^{2+} concentration and with eGFR are observed in ADPKD patients with normal renal function as well as in other patients with early renal failure. This may indicate that the natural course of ADPKD leads to calcium metabolism disorders before the onset of renal failure. An elevated Ca^{2+} concentration in erythrocytes of ADPKD patients with normal renal function may be the result of a dysfunction of mutated polycystins. Its value as a potential prognostic factor requires further research.

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