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Renal αKlotho expression is associated with myocardial hypertrophy (experimental study)

E. O. Bogdanova[1], N.Yu. Semenova[3], O. N. Beresneva[1], M. M. Parastaeva[1], G. T. Ivanova[4], O. V. Galkina[1], I. M. Zubina[1], I. G. Kayukov[1], T. L. Kovalenko[2], L. V. Kotenko[2], V. A. Dobronravov[1]

[1] Research Institute of Nephrology at First Pavlov State Medical University of St. Petersburg, St Petersburg, Russia
[2] Center of Laboratory Diagnostics of First Pavlov State Medical University of St. Petersburg, St Petersburg, Russia
[3] Russian Research Institute of Hematology and Transfusiology, St Petersburg, Russia
[4] Pavlov Institute of Physiology of Russian Academy of Sciences, St Petersburg, Russia

Corresponding author:
Evdokiya O. Bogdanova,
Research Institute of Nephrology, First Pavlov State Medical University of St. Petersburg, 17 L. Tolstoy street, St Petersburg, 197101 Russia.
Phone: +7(812)234–66–56.
Fax: + 7(812)234–95–63.
E-mail: evdokia.bogdanova@gmail.com

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Abstract

Objective. The study was aimed to test a hypothesis on possible connection between αKlotho and (or) fibroblast growth factor 23 (FGF23) and myocardial hypertrophy at early stages of the renal dysfunction (RD). **Design and methods.** Experimental models of chronic kidney injury were 3/4 or 5/6 nephrectomy (NE) in SHR rats. Sham-operated SHR rats were used as control. The timing of experiments was one or two months to achieve an expected fall of glomerular filtration rate (GFR) corresponding to early stages of RD. αKlotho protein in tubular epithelium was detected by immunohistochemistry. Serum concentrations of FGF23 and intact parathyroid hormone (iPTH), serum and urine levels of inorganic phosphate (Pi), Na, creatinine and protein as well as myocardial mass index (MMI) were measured. **Results**. Implemented models of RD corresponded to 1C–3C stages of human chronic kidney disease. Renal excretion of Pi significantly increased in the groups of nephrectomized animals. No significant differences were observed in serum concentrations of FGF23 and iPTH whereas the renal αKlotho expression decreases along with an increasing degree of kidney injury and MMI. The significant negative association between MMI and the renal αKlotho expression was independent of other potential confounders as confirmed by a multivariate regression analysis. **Conclusions.** The obtained experimental data suggest that αKlotho can participate in mechanisms of myocardial remodeling in persistent hypertension and RD.

Key words: renal dysfunction, arterial hypertension, myocardial mass index, αKlotho, fibroblast growth factor 23

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Почечная экспрессия белка αKlotho ассоциирована с гипертрофией миокарда (экспериментальное исследование)

Е. О. Богданова[1], О. Н. Береснева[1], Н. Ю. Семенова[3], М. М. Парастаева[1], Г. Т. Иванова[4], О. В. Галкина[1], И. М. Зубина[1], И. Г. Каюков[1], Т. Л. Коваленко[2], Л. В. Котенко[2], В. А. Добронравов[1]

[1] Научно-исследовательский институт нефрологии Государственного бюджетного образовательного учреждения высшего профессионального образования «Первый Санкт-Петербургский государственный медицинский университет имени академика И. П. Павлова» Министерства здравоохранения Российской Федерации, Санкт-Петербург, Россия
[2] Центр лабораторной диагностики Государственного бюджетного образовательного учреждения высшего профессионального образования «Первый Санкт-Петербургский государственный медицинский университет имени академика И. П. Павлова» Министерства здравоохранения Российской Федерации, Санкт-Петербург, Россия
[3] Лаборатория по изучению лейкозов Российского научно-исследовательского института гематологии и трансфузиологии, Санкт-Петербург, Россия
[4] Институт физиологии имени И. П. Павлова Российской академии наук, Санкт-Петербург, Россия

Контактная информация:
Богданова Евдокия Олеговна,
НИИ нефрологии, ГБОУ ВПО СПбГМУ им. И.П. Павлова Минздрава России, ул. Л. Толстого, д. 17, Санкт-Петербург, Россия, 197101.
Тел.: +7(812)234–66–56.
Факс: +7(812)234–95–63.
E-mail: evdokia.bogdanova@gmail.com

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Резюме

Цель исследования. Целью выполненного исследования была экспериментальная проверка гипотезы о наличии возможной связи между изменениями в системе фактор роста фибробластов 23 (FGF23)/αKlotho и прогрессированием гипертрофии миокарда на ранних стадиях формирования дисфункции почек (ДП). **Материалы и методы.** Для моделирования ДП выполняли резекцию 3/4 и 5/6 почечной ткани у крыс линии SHR, сроки эксперимента — 1 и 2 месяца. В качестве контроля использовали ложнооперированных животных. Анализировали индекс массы миокарда (ИММ), содержание белка αKlotho в тубулярном эпителии (иммуногистохимическим методом), концентрации FGF23, интактного паратиреоидного гормона (iPTH) в сыворотке крови (иммуноферментный анализ), а также неорганического фосфата (Pi), натрия, креатинина в сыворотке крови и моче, концентрации белка в моче. **Результаты.** Реализованные модели соответствуют клиническим стадиям 1–3 хронической болезни почек. Почечная экскреция Pi увеличивается в группах животных, подвергнутых нефрэктомии. По мере нарастания степени повреждения почек и увеличения ИММ существенных изменений концентраций FGF23 и iPTH в сыворотке крови не выявлено, в то время как содержание белка αKlotho в почке значительно снижалось. При мультивариантном анализе показана значимая обратная связь между ИММ и содержанием αKlotho в почке, независимая от влияния других исследуемых факторов, включая уровень артериального давления и степень снижения функции почек. **Выводы.** Содержание белка αKlotho в тубулярном эпителии почки ассоциировано с ИММ, что позволяет предположить участие

αKlotho в механизмах ремоделирования миокарда в условиях персистирования артериальной гипертензии и ДП.

Ключевые слова: дисфункция почек, артериальная гипертензия, индекс массы миокарда, αKlotho, фактор роста фибробластов 23

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Introduction

Patients with chronic kidney disease (CKD) are at high of developing cardiovascular diseases that are the predominant cause of mortality in end-stage CKD [1–4]. An imbalance of inorganic phosphate (Pi) is common in metabolic disorders in progressive renal dysfunction (RD). The recent experimental and clinical studies proved the relationship between Pi-related factors and cardiovascular risk: calcification of the aorta, coronary arteries and peripheral vascular disease, left ventricular hypertrophy and mortality in CKD [4–10]. Over the last decade, the understanding of the endocrine regulation of Pi metabolism and its violations in CKD has broadened considerably. It is due to the discovery of phosphotonic system including fibroblast growth factor 23 (FGF23) and its co-receptor — a protein αKlotho [11, 12]. The reaction of FGF23 / αKlotho in response to Pi retention is believed to precede the change of "classical" phosphate-regulating factors — calcitriol and parathyroid hormone (PTH) [13, 14]. Some data also suggests a relation between increased plasma level of FGF23 and cardiovascular complications in patients with CKD [15, 16] and in general population [17, 18]. However, αKlotho may have FGF23-independent effects on the cardiovascular system [7, 19–21]. The majority of the cited experimental and clinical studies assessed the effects in severe PD, while the renal and systemic changes in Pi metabolism and its regulation occur in the early stages of chronic kidney damage.

The aim of the study was an experimental verification of the hypothesis of a potential link between changes in the FGF23 / αKlotho system and progression of cardiac hypertrophy (CH) in the early stages of PD.

Table 1

CHRONIC KIDNEY DAMAGE MODEL AND EXPERIMENT TERMS

Parameter	Terms of the experiment, group indexation, n	
Description of the model	1 month	2 months
Control	K(1), 9	K(2), 9
nephrectomy 3/4	3/4NE(1), 9	3/4NE(2), 10
nephrectomy 5/6	5/6NE(1), 9	5/6NE(2), 8

Design and methods

Experimental model

Adult male SHR rats (the farm kennel "Koltushi" RAN) weighing 190–230 grams were used. The animals were kept under standard vivarium conditions in the Institute of Physiology named after I. P. Pavlov of Russian Academy of Sciences. Experimental PD was modeled by nephrectomy (NE) of the 3/4 [22] or 5/6 [23, 24] of the kidney volume. The experiment duration was 1 and 2 months (Table 1).

During the experiment, the animals got standard laboratory diet containing 0.8% phosphates and had free access to water. Before completion of the experiment the animals were placed in a metabolic chamber for 24 hours; during this period 24-hour urine was collected, and blood pressure (BP) was measured by the previously described procedure [26]. Before the experiment was completed, the myocardial mass index (MMI) was calculated as a ratio of kidney weight to body weight (mg/g), and kidney and blood samples were collected [25]. The blood was centrifuged at 1000 g for 30 minutes, and aliquot of serum and urine were stored at –80 ° C. Experiments were carried out in accordance with the requirements for the use of laboratory animals, after approval by local ethics committee.

Histological studies

For light-optical microscopy parenchyma (1–2 mm) from the middle of the left kidney was fixed in 5 % formalin prepared with PBS (pH 7.2) for 16 hours, at room temperature. Sections not more than 5 microns in thickness were placed on a glass coated with polylysine, paraffin was eliminated, the ectiones were hydrated, and antigen was unmasked by standard procedures. Endogenous peroxidase activity was eliminated by exposure to a peroxidase-blocking solution for 6 minutes (Spring Bioscience, USA). Primary polyclonal anti-Klotho rabbit antibodies (Abcam, UK) were used for immunohistochemical reaction at a dilution of 1: 250. The system "REVEAL-Biotin-Free Polyvalent DAB" (Spring Bioscience, USA) was used for assessment. Morphometric analysis was performed with the use of the software "VideoTesT-Morphology 5.2". The average area of the specific product of the immunohistochemical reaction of the total tubulointerstitium area in 10 fields of vision was assessed.

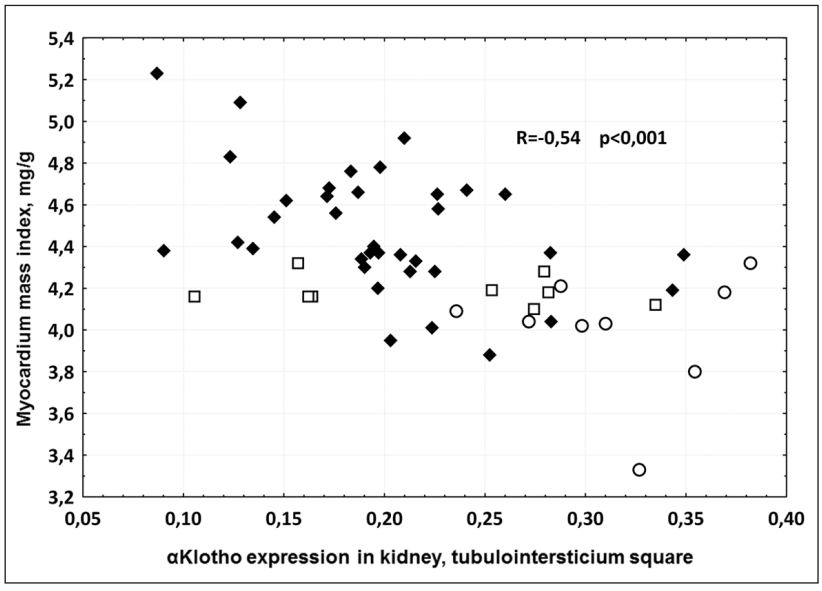
Enzyme immunoassay and biochemical studies

Serum FGF23 level was assessed by the test system "FGF23 ELISA Kit" (Kainos Laboratories, Inc., Japan). The intact PTH (iPTH) level was measured using the test system "Rat Intact PTH ELISA Kit" (Immutopics, Inc., USA). Optical density was assessed by a semi-automatic microplate analyzer "ImmunoChem 2100" (High Technology, USA). The levels of Pi, sodium (Na), creatinine in serum and urine, and urinary protein concentration were measured by standard methods with the use of an automated analyzer "SYNCHRON CX DELTA" (Beckman-Soulter, USA). Fractional excretion of Pi (FEPi) and Na (FENa) was calculated using the formula: FE (%) = (Ux x SCr)/(Sx x UCr) x 100, where Ux is the urine concentration of Pi / Na, Sx — serum concentration of Pi / Na, UCr — the urine concentration of creatinine, and SCr — serum creatinine concentration. Also 24-hour urinary excretion of Pi was assessed by the formula: UPi24 (mmol) = D x UPi, UPi, where UPi is the urine concentration of phosphorus, and D is the 24-hour diuresis. The daily proteinuria was assessed by the ration urine protein/creatinine (PCR).

Statistical analysis

Data are presented as mean and standard deviation (± SD) or median and interquartile range. Two-sided t-test or the Mann-Whitney test were used to compare the mean values in two samples. Spearman correlation and multiple linear regression analysis were used to assess the relationship between αKlotho and MMI. In the regression anal-

Figure. The association between myocardium mass index and αKlotho level in kidney in progressive chronic kidney disease in SHR rats



Note: Correlation coefficient for the joined group of the animals (n = 54); white circles — control group K(1); white squares — control group K(2); black rhombus — joined group of nephrectomized animals (NE), 3/4NE(1) + 5/6NE(1) + 3/4NE(2) + 5/6NE(2).

ysis MMI was considered as the dependent variable, and αKlotho renal concentration and other covariates were included as independent variables. The significance level for all statistical tests was set at 0.05.

Results

As the severity of chronic kidney damage enhanced, the relative increase in creatinine level ranged from 30% to 60% in experimental groups compared to controls. Thus, the models corresponded to the clinical CKD stages 1–3 [27]. The modeling of renal dysfunction led to regular changes, in particular, to an increase in urinary protein and FEN excretion. Serum Pi level decreased while FEPi and UPi24 increased in animals at 1 and 2 months after nephrectomy compared to control groups (Tables 2 and 3). At different stages of the experiment, a natural tendency to BP and MMI increase was noted in nephrectomized animals compared to controls. When comparing the groups of animals at the same period of experimental exposure the concentrations of the phosphotonic factors (iRTN and FGF23) did not differ significantly. However, αKlotho level in the kidney distinctly decreases as degree of renal damage and MMI increase (Tables 2 and 3).

The analysis of the combined group of experimental animals demonstrated a significant inverse correlation between MMI and αKlotho concentration in the renal tubular epithelium (Fig.).

Multiple regression analysis confirmed a significant relationship between renal αKlotho concentration and MMI, after adjustment for the other independent variables that could potentially influence the development of the myocardial hypertrophy, including BP and SCr (Table 4).

Discussion

In our study, the model of myocardial hypertrophy included two factors — elevated BP and experimental chronic RD in SHR rats [28]. Left ventricular hypertrophy (LVH) is known to be significantly more prevalent in CKD patients compared to the general population [15], mainly due to hemodynamic factors: hypertension [29],

Table 2

CHARACTERISTICS OF THE ANIMALS AT ONE MONTH OF EXPERIMENTS

Parameter	Control, K (1)	NE 3/4 (1)	NE 5/6 (1)	p_{1-2}	p_{2-3}	p_{1-3}
	1	2	3			
Body weight, g	317 ± 24	285 ± 19	289 ± 8	0,007	0.56	0.005
Systolic blood pressure, mm Hg	196 ± 9	197 ± 9	205 ± 9	0,80	0.072	0.039
Daily proteinuria, g/100 g of body weight	0.165 ± 0.019	0.134 ± 0,048	0.243 ± 0.055	0.090	< 0.001	0.001
SCr, mmol/100 g body weight	0.011 ± 0.003	0.019 ± 0.002	0.025 ± 0.004	< 0.001	< 0,001	< 0.001
MMI, mg/g	4.00 ± 0.29	4.29 ± 0.21	4.61 ± 0.33	0.028	0.028	0.001
αKlotho concentration in the kidney, tubulointerstitium area	0.32 ± 0.05	0.24 ± 0.07	0.20 ± 0.05	0.018	0.129	< 0.001
FGF23, pg/ml	548 ± 241	650 ± 475	782 ± 299	0,58	0.49	0.087
iPTH, pg/ml	87 (74; 126)	87 (64; 109)	148 (136; 165)	0.79	0,11	0.094
FENa, %	0.69 ± 0.32	0.67 ± 0.18	1.11 ± 0.23	0.85	< 0.001	0.006
SPi, mmol/l	2.54 ± 0.13	2.43 ± 0.15	2.27 ± 0.13	0.13	0.031	< 0.001
FEPi, %	17 ± 8	23 ± 5	38 ± 8	0,080	< 0,001	< 0.001
UPi_{24}, mmol	0.61 ± 0.25	0.89 ± 0.16	0.94 ± 0.19	0.016	0.41	0.006

Note: SCr — serum creatinine; MMI — myocardial mass index; FGF23 — fibroblast growth factor 23; iPTH — intact parathyroid hormone; FENa — fractional excretion of Na; SPi — inorganic phosphate serum; FEPi — fractional excretion of inorganic phosphate; UPi24 — daily urinary excretion of inorganic phosphate.

Table 3

CHARACTERISTICS OF THE ANIMALS AT TWO MONTHS OF EXPERIMENT

Parameter	Control, K (2)	NE 3/4 (2)	NE 5/6 (2)	p_{1-2}	p_{2-3}	p_{1-3}
	1	2	3			
Body weight, g	320 ± 17	297 ± 21	328 ± 19	0.018	0.006	0.42
Systolic blood pressure, mm Hg	190 ± 10	204 ± 17	210 ± 10	0.043	0.39	0.001
Daily proteinuria, g/100 g of body weight	0.136 ± 0.028	0.461 ± 0.160	0.604 ± 0.199	< 0.001	0.11	< 0.001
SCr, mmol/100 g of body weight	0.011 ± 0.001	0.020 ± 0.004	0.021 ± 0.002	< 0.001	0.86	< 0.001
MMI, mg/g	4.19 ± 0.07	4.41 ± 0.29	4.61 ± 0.25	0.044	0.13	< 0.001
αKlotho concentration in the kidney, tubulointerstiTIAL	0.22 ± 0.08	0.21 ± 0.03	0.13 ± 0.02	0.71	< 0.001	0.007
FGF23, pg/ml	543 ± 170	723 ± 211	1013 ± 720	0.058	0.24	0.076
iPTH, pg/ml	37 (22; 105)	80 (13; 140)	95 (80; 123)	0.60	0.36	0.11
FENa, %	0.24 ± 0.07	0.57 ± 0.22	0.55 ± 0.10	< 0.001	0.83	< 0.001
SPi, mmol/l	2.64 ± 0.36	2.24 ± 0.20	2.14 ± 0.23	0.011	0.07	< 0.001
FEPi, %	11 ± 2	27 ± 8	35 ± 5	< 0.001	0.017	< 0.001
UPi_{24}, mmol	0.49 ± 0.20	0.98 ± 0.13	1.04 ± 0.11	< 0.001	0.32	< 0.001

Note: SCr — serum creatinine; MMI — myocardial mass index; FGF23 — fibroblast growth factor 23; iPTH — intact parathyroid hormone; FENa — fractional excretion of Na; SPi — inorganic phosphate serum; FEPi — fractional excretion of inorganic phosphate; UPi24 — daily urinary excretion of inorganic phosphate.

Table 4

THE ASSOCIATION BETWEEN AKLOTHO LEVEL IN THE KIDNEYS AND BODY MASS INDEX

Covariates	Model 1[a]	Model 2[b]	Model 3[c]	Model 4[d]	Model 5[e]	Model 6[f]
Independent factor	4.50 ± 0.80 (< 0.001)	4.51 ± 0.75 (< 0.001)	4.55 ± 0.71 (< 0.001)	4.40 ± 0.70 (< 0.001)	4.34 ± 1.08 (< 0.001)	4.45 ± 0.84 (< 0.001)
αKlotho concentration in the kidney	–1.97 ± 0.75 (0.012)	–1.54 ± 0,62 (0.018)	–2.43 ± 0.85 (0.012)	–1.52 ± 0.63 (0.019)	–1.99 ± 0.76 (0.013)	–1.94 ± 0.78 (0.017)

Note: The regression coefficients B ± SD are given, p-values are given in parenthesis; a — independent variables in the model 1: αKlotho concentration in the kidney, serum creatinine, blood pressure, daily proteinuria; b — independent variables in the model 1 + FEPi; c — independent variables in the model 1 + FENa; d — independent variables in the model 1 + UPi24; e — independent variables in the model 1 + SPi; f — independent variables in the model 1 + FGF23 + iRTN.

anemia [30], and fluid retention [31]. Given the numerous systemic metabolic and endocrine changes, developing in progressive CKD, we assume the existence of additional mechanisms associated with the development of myocardial hypertrophy. In particular, this applies to systemic disorders of phosphate metabolism, including Pi retention due to the abnormal renal excretion and phosphotonic dysregulation. The latter one manifests as an increased synthesis of FGF23 and PTH, and renal and extrarenal αKlotho deficiency [7, 10, 19, 32, 33].

Our study shows that in a model of the early stages of chronic kidney damage renal αKlotho protein is associated with an increase in MMI. We can hypothesize that the relation between αKlotho and MMI demonstrates their collinearity as both depend on RD severity. However, multiple regression analysis did not confirm its dependence on the traditional risk factors — high BP and RD severity. We assume that the decline in αKlotho level in RD may be a different mechanism leading to the additional progression

of LVH in rats SHR with pre-existing chronic hypertension.

A number of experimental and clinical studies demonstrate a link between other factors common in cases of abnormal phosphate metabolism and left ventricular hypertrophy — hyperphosphatemia [34], PTH [34–36] and FGF23 [6, 10, 18, 37]. At the same time, in this study the potential impact of these factors on the myocardium was minimal due to the modeling of early stages of RD. The impact of hyperphosphatemia was excluded, because in experimental animals with higher MMI serum Pi levels decreased significantly with the progression of RD due to the increased absolute and relative urinary PI excretion. In animals after nephrectomy, there was no increase in concentrations of FGF23 and iRTN inherent in advanced CKD, which corresponds to the estimated glomerular filtration rate lower than 40 mL/min in humans. Calcitriol is another significant factor for the development of Pi imbalance and cardiovascular changes in RD [5, 38]. We did not measure circulating calcitriol level that is one of the limitations of this study. However, the reduced production of the active D-hormone results from the regulatory effect of FGF23 on the kidney [39]. Thus, FGF23 might be involved in this feedback mechanism regulating cardiac hypertrophy progression.

Potential cardiac effects of αKlotho can be divided into direct ones and indirect ones involving remodeling of the arterial wall. αKlotho deficiency is known to be associated with vascular dysfunction [40] and arterial calcification [7, 34, 36]. Experimental studies with knockout and transgenic animals showed that αKlotho acts as an inhibitor of calcification [19]. Arterial calcification and stiffness is a known factor of pulse pressure changes and left ventricular hypertrophy [41]. However, this mechanism plays role in LVH development in long persistent RD (for example, in dialysis patients), but it is unlikely in experiments with short-term RD.

The convincing evidence of αKlotho synthesis in cardiomyocytes (CMC) is lacking. So the mechanisms of its cardiac effects are disputable. The kidney is known to be the major site of circulating αKlotho production. After secretion, the latter enters the circulation and independently affects extrarenal cell populations [42–44]. The link between renal αKlotho and MMI suggests an important role of circulating αKlotho, although the potential paracrine effects of αKlotho produced in vascular smooth muscle cells should be also considered.

Only two papers consider the possible direct impact of αKlotho on CMC and its role in myocardial remodeling [20, 21]. One suggested that the reduction in number of transient receptor potential channel in CMC mediates the inhibiting effect of αKlotho on LVH. Calcium is a secondary messenger in the signaling pathway of calcineurin, and intracellular calcium transport through TRPC6 ion channels appears to play an essential role in the development of cardiac hypertrophy. Overexpression of the gene *Trpc6* results in a spontaneous cardiac hypertrophy in mice, while overexpression of *KL* gene (which encodes αKlotho) prevents this effect. At the same time, *Trpc6* deletion prevents the myocardial mass growth in mice with αKlotho deficiency. Glucosidase activity of αKlotho modifies the carbohydrate component of ion channels and is not involved in the regulation of TRPC6 [20]. The suggested mechanism is the inhibition of IGF1 (Insulin-like growth factor I) and PI3K (Phosphatidylinositol-4, 5-bisphosphate 3-kinase) dependent exocytosis of TRPC6 in CMC [20]. By the same mechanism circulating αKlotho reduces the consequences of oxidative stress independently of FGF23. Moreover, αKlotho prevents CMC apoptosis increasing phosphorylation of pro-apoptotic factors JNK (c-Jun NH2-terminal kinase), and p38 [21].

Thus, our study demonstrates an association between the concentration of αKlotho in kidney tubular epithelium and MMI, suggesting the role of αKlotho in cardiac remodeling in persistent hypertension and RD.

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Conflict of interest

The authors declare no conflict of interest.

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Author information:

Evdokiya O. Bogdanova, Junior Researcher, Research Institute of Nephrology, First Pavlov State Medical University of St. Petersburg;

Olga N. Beresneva, PhD, Biology Sciences, Senior Researcher, Center of Laboratory Diagnostics, First Pavlov State Medical University of St. Petersburg;

Natalya Yu. Semenova, Junior Researcher, Russian Research Institute of Hematology and Transfusiology;

Marina M. Parastaeva, PhD, Biology Sciences, Senior Researcher, Laboratory of Clinical Physiology of Kidneys, Research Institute of Nephrology, First Pavlov State Medical University of St. Petersburg;

Galina T. Ivanova, PhD, Biology Sciences, Senior Researcher, Pavlov Institute of Physiology of Russian Academy of Sciences;

Olga V. Galkina, PhD, Biology Sciences, Associate Professor, Head, Laboratory of Biochemical Homeostatis, Research Institute of Nephrology, First Pavlov State Medical University of St. Petersburg;

Irina M. Zubina, PhD, Biology Sciences, Senior Researcher, Laboratory of Biochemical Homeostatis, Research Institute of Nephrology, First Pavlov State Medical University of St. Petersburg;

Ivan G. Kayukov, MD, PhD, DSc, Professor, Head, Laboratory of Clinical Physiology of Kidneys, Research Institute of Nephrology, First Pavlov State Medical University of St. Petersburg;

Tatyana L. Kovalenko, Center of Laboratory Diagnostics, First Pavlov State Medical University of St. Petersburg;

Larisa V. Kotenko, Head, Biochemical Laboratory, Center of Laboratory Diagnostics, First Pavlov State Medical University of St. Petersburg;

Vladimir A. Dobronravov, MD, PhD, DSc, Professor, Department of the Propaedeutics of Internal Diseases, Deputy Director on Research, Research Institute of Nephrology, First Pavlov State Medical University of St. Petersburg.