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The Expression and Implication of TRPV5, Calbindin-D28k and NCX1 in Idiopathic Hypercalciuria

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Summary：The expression of calcium epithelium TRPV5, alcium binding protein Calbindin-D28k and Na^+/Ca^{2+} exchanger NCX1 was detected in renal distal convoluted tubule, and their effects on urine calcium reabsorption and the possible pathogenic mechanism in idiopathic hypercalciuria (IH) were investigated. Genetic hypercalciuric stone-forming (GHS) rats were chosen as animal models to study urine calcium reabsorption and IH. The cognate female and male rats that had maximal urine calcium were matched to breed next generation. Twelve GHS rats and 12 normal control (NC) SD rats were selected. Western blot and real time quantitative PCR were used to detect the protein and gene expression of TRPV5, Calbindin-D28k and NCX1 respectively. The expression levels of TRPV5 protein and mRNA in GHS rats were significantly lower than in NC rats (P<0.05). Western blot revealed that the expression levels of Calbindin-D28k in GHS rats and NC rats were 0.49±0.02 and 0.20±0.01 respectively, with the difference being significant between them (P<0.05). By using real time quantitative PCR, it was found that there was no significant difference in Calbindin-28k mRNA expression levels between GHS rats and NC rats (P>0.05). There was no significant difference in the NCX1 expression between GHS rats and NC rats (P>0.05). It was suggested that TRPV5 and Calbindin-D28k might play an important role in urine calcium reabsorption and IH, but they differently contributed to the pathogenesis: The down-regulation of TRPV5 decreases urine calcium reabsorption, directly leading to loss of the urine calcium and resulting in hypercalciuria, and the increased Calbindin-D28k expression could relieve, neutralize and decrease intracellular Ca^{2+} concentration to maintain calcium balance. NCX1 is not the key protein in urine calcium reabsorption.
Key words：TRPV5; Calbindin-D28k; NCX1; idiopathic hypercalciuria; reabsorption; VDR

Idiopathic hypercalciuria (IH) is an important risk factor of urolithiasis, however, the pathogenesis of IH is unknown. Urine calcium reabsorption was considered to be a consecutive transcellular process. TRPV5, Calbindin-D28k and NCX1 are significant proteins which participate in urine calcium reabsorption, and all of them distribute in renal distal convoluted tubule and collecting tubule[1, 2]. TRPV5, which was called ECAC1 previously, is mainly expressed in lumina side of epithelial cells and reabsorbs the calcium in lumina. Calbindin-D28k, which locates in the same site as TRPV5, can combine and transport endochylema Ca^{2+}. NCX1 distributes in basilemma of epithelial cells and can discharge the endochylema Ca^{2+}. Genetic hypercalciuric stone-forming (GHS) rats have the similar alteration as human, so GHS rats are regarded as the most ideal animal models[3]. This study intended to illuminate the possible effects of TRPV5, Calbindin-D28k and NCX1 respectively on urine calcium reabsorption, and investigate their implications in the pathogenesis of IH.

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1 MATERIALS AND METHODS

1.1 Breeding of GHS Rats and Experiment Grouping

Cognate female and male SD rats whose urine calcium were maximal were matched to breed next generation, and their steady hereditary features of IH appeared from the 7th generation[3]. Twelve clean and 3-month-old GHS rats at 12th generation were selected and characterized by the follows: the 24-h urine calcium exceeding 1.5 mg in normal diet, but blood calcium, $1\alpha,25(OH)_2D_3$ and parathyroid hormone were in the normal range; renal stones could be observed under a microscope and mostly distributed in medulla; Stone ingredient test demonstrated that combination calculi in GHS rats were mainly composed of calcium phosphate stone; possibility of crystallization was eliminated by further observation and test. Twelve normal control rats which were body weight- and age-matched with GHS rats were bought from Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology (China).

1.2 Main Reagents

Rabbit anti-rat TRPV5 primary antibody (Abcam, USA), rabbit anti-rat Calbindin-D28k primary antibody (Santa Cruz, USA), rabbit anti-rat NCX1 primary anti-

body (Santa Cruz, USA), goat anti-rabbit secondary antibody (Wuhan Jingmei Co. Ltd, China), Trizol (Invitrogen, USA), cDNA synthesis kit and SYBR Green Ⅰ Premix (Dalian TaKaRa, China).

1.3 Collection of Specimens

Rats in NC and GHS groups were killed under anesthesia with pentobarbital. Kidney tissues were removed under aseptic condition, immediately frozen in liquid nitrogen and cut into pieces of 50–100 mg.

1.4 Western Blot Analysis

The pieces of tissue were homogenized by a potter homogenizer on ice and the protein concentrations of TRPV5, Calbindin-D28k and NCX1 were determined by ultraviolet spectrophotometer. To evaluate the abundance of TRPV5, 100 μg of proteins obtained from two groups were diluted in 5×loading buffer and separated on 8% SDS-PAGE. After electrophoresis, all gels were submitted to electroblotting to transfer the proteins on a nitrocellulose membrane that was washed with Tris-buffered saline (TBS, 150 mmol/L NaCl, 100 mmol/L Tris-HCl, pH 7.5). After the wash, the membrane was incubated with 5% defatted milk for 1 h and then probed for 1 h with a rabbit anti-rat TRPV5 primary antibody diluted to 1:1000. Secondary goat anti-rabbit IgG horseradish peroxidase conjugate was diluted to 1:5000 and incubated with blocking buffer for 1 h. At last, the nitrocellulose membrane was performed three times with TEST (1 g/L Tween 20 in TBS). The final detection was obtained with enhanced chemiluminescence method in dark room and the picture was taken. Similar methods or procedures were performed for NCX1. To Calbindin-D28k, the similar methods were used as above except for 12% SDS-PAGE; rabbit anti-rat Calbindin-D28k primary antibody (1:100) and Diamonobenzidine (DAB) detection method. All proteins including TRPV5, Calbindin-D28k, NCX1 and internal standard (β-actin) were quantified by Quantity One 4.6 analysis software (Bio-Rad, USA), and the ratio of proteins and β-actin was marked as *A* which represented semiquantitative value respectively.

1.5 Real Time Fluorescent Quantitative Polymerase Chain Reaction

TRPV5 primers: sense, 5'-CTTACGGGTTGAACACCACCA-3'; antisense, 5'-TTGCAGAACCACAGAGCCTCTA-3'. Calbindin-D28k primers: sense, 5'-GGAAGCTGGAGCTGACAGAGAT-3'; antisense, 5'-TGAACTCTTTCCCACACATTTTGAT-3'. NCX1 primers: sense, 5'-CCCAGGACCAGTATGCAGAT-3'; antisense, 5'-CATGGTAGATGGCAGCAATG-3'. GAPDH primers: sense, 5'-GCCAGCCTCGTCTCATAGACA-3'; antisense, 5'-AGAGAAGGCAGCCCTGGTAAC-3'. Total RNA was extracted from kidney tissues using Trizol Total RNA Isolation Reagent according to the manufacturer's protocol. The RNA absorbance (*A*) was measured by ultraviolet spectrophotometer and the range of *A*260/*A*280 was from 1.8 to 2.0. Total RNA (2 μg) was subjected to reverse transcription using Moloney Murine Leukemia Virus reverse transcriptase. Expression levels of TRPV5, Calbindin-D28k and NCX1 were quantified by real-time quantitative PCR using Roto Gene 3000 sequence detector (Corbett, Aus). PCR reactions were performed in a total volume of 20 μL in the presence of 10 μL of 2 ×Premix SYBR Green Ⅰ, 0.4 μL of each primer, 2 μL cDNA, 7.2 μL H_2O. Samples were firstly denatured at 95°C for 10 s, and followed by 40 cycles consisting of denaturing at 95°C (5 s), annealing at 60°C (20 s). Fluorescence value was measured after annealing and molten curve analysis was run according to default condition. Goal gene and GAPDH gene were subjected to the PCR under the same reaction conditions and negative control was set up simultaneously. The C_T value represented PCR cycle times when fluorescence signal reached threshold from basic to exponential growth stage, and it could be calculated by Roto Gene 3000 software. The less C_T value was calculated, the higher mRNA expression level was represented. The difference value (ΔC_T) could be obtained using respective goal gene (TRPV5, Calbindin-D28k and NCX1) C_T and GAPDH C_T in two groups and the difference value ($\Delta\Delta C_T$) which represented ΔC_T of GHS rats minus ΔC_T of NC rats. Finally, the data were analyzed using $2^{-(\Delta\Delta CT)}$ method. The results represented the times that gene expression in GHS group vs NC group.

1.6 Statistical Analysis

The data were expressed as $\bar{x}\pm s$ and analyzed using SPSS12.0 software, and paired Student's *t*-test was used for statistical comparison.

2 RESULTS

2.1 Measurement of TRPV5 Protein Abundance by Western Blot

It was shown that TRPV5 antibody recognized an 85-kD protein. Results which obtained by densitometric analysis demonstrated that TRPV5 abundance (*A*) was 0.30±0.05 (n=12) in GHS rats, while it was increased to 0.59±0.08 (n=12) (P<0.05) in NC rats (fig. 1).

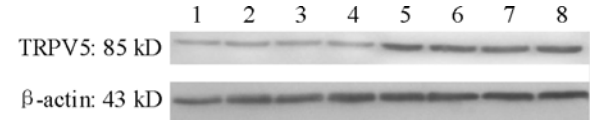

Fig. 1 Western blot results for TRPV5 expression in renal cortex of GHS rats (1–4) and NC rats (5–8)

2.2 Measurement of Calbindin-D28k Protein Abundance by Western Blot

Fig. 2 represented the expression of Calbindin-D28k in NC membrane. Western blot showed that Calbindin-D28k abundance in NC rats (0.20±0.01) was weaker than in GHS rats (0.49±0.02) (P<0.05).

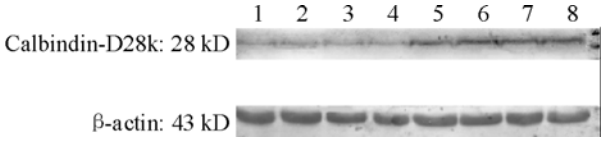

Fig. 2 Expression of Calbindin-D28k in NC rats (1–4) and GHS rats (5–8)

2.3 Measurement of NCX1 Protein Abundance by Western Blot

Fig. 3 represented the expression of NCX1 in GHS rats and NC rats. Western blot showed that NCX1 abundance in GHS rats and NC rats was 0.35±0.03 and 0.36±0.04 with the difference being not significant (P>0.05).

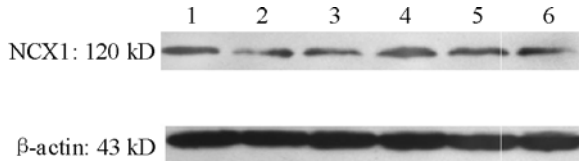

Fig. 3 Expression of NCX1 protein in GHS rats (1–3) and NC rats (4–6)

2.4 Expression of TRPV5, Calbindin D28k and NCX1 mRNA

Tables 1, 2 and 3 represented the expression levels of TRPV5, Calbindin D28k and NCX1 mRNA respectively. Primer dimer and specific amplification were not detected in molten curve analysis. Each gene product was unimodal, and no fluorescence was found in negative control group. The C_T values of TRPV5 and GAPDH in two groups were shown in table 1. $2^{-(\Delta\Delta C_T)}$ results demonstrated that TRPV5 mRNA abundance in NC rats was 11.9 times higher than in GHS rats (P<0.01). The C_T values of Calbindin D28k and GAPDH in two groups were shown in table 2. $2^{-(\Delta\Delta C_T)}$ results demonstrated that Calbindin-D28k mRNA abundance in GHS rats was 1.21 times higher than in NC rats with the difference being not significant (P>0.05). As well, the C_T values of NCX1 and GAPDH in two groups were shown in table 3, and there was no significant difference between two groups (P>0.05).

Table 1 TRPV5 mRNA in kidney of GHS and NC rats ($\bar{x}$±s, n=12)

Groups	TRPV5 Mean C_T	GAPDH Mean C_T	ΔC_T TRPV5- GAPDH	$\Delta\Delta C_T$ ΔC_T.GHS-ΔC_T.NC	$2^{-(\Delta\Delta C_T)}$ TRPV5.GHS/NC
NC	23.12±1.01	20.76±0.93	2.36±0.22	0	1.0
GHS	25.77±0.51	19.84±0.22	5.93±0.31	3.57±0.31	0.08 (0.07–0.1)

$2^{-(\Delta\Delta C_T)}$: TRPV5 gene expression times in GHS rats vs NC rats

Table 2 Calbindin-D28k mRNA in kidney of GHS and NC rats ($\bar{x}$±s, n=12)

Groups	CaBP-28KD Mean C_T	GAPDH Mean C_T	ΔC_T CaBP28KD-GAPDH	$\Delta\Delta C_T$ ΔC_T.GHS-ΔC_T.NC	$2^{-(\Delta\Delta C_T)}$ CaBP28KD.GHS/NC
NC	22.45±0.96	21.25±0.51	1.20±0.52	0	1.0
GHS	20.39±0.86	19.46±0.75	0.92±0.43	–0.28±0.31	1.21 (0.98–1.51)

$2^{-(\Delta\Delta C_T)}$: Calbindin-D28k gene expression times in GHS rats vs NC rats

Table 3 NCX1 mRNA in kidney of GHS and NC rats ($\bar{x}$±s, n=12)

Groups	NCX1 Mean C_T	GAPDH Mean C_T	ΔC_T NCX1- GAPDH	$\Delta\Delta C_T$ ΔC_T.GHS-ΔC_T.NC	$2^{-\Delta\Delta C_T}$ NCX1.GHS/NC
NC	23.71±0.45	16.96±0.47	6.75	0	1.0
GHS	23.25±0.64	16.62±0.29	6.63	–0.12	1.09

$2^{-(\Delta\Delta C_T)}$: NCX1 gene expression times in GHS rats vs NC rats

3 DISCUSSION

Half of patients with urinary calculus are caused by IH[4]. The pathogenesis of IH is not clear. GHS rats have identical pathological and physiological changes with IH patients: Under normal diet, the serum calcium and 1α,25$(OH)_2D_3$ are in the normal range but absorption of calcium in gastrointestinal tract is increased; bone density and urine calcium reabsorption in renal tubule are decreased and urine calcium excretion is obviously increased, resulting in the formation of renal calculus; hypercalciuria and calculi are determined by hereditary factor rather than environmental factors and other pathological alterations that can be detected *in vitro*. Therefore, GHS rats are considered to be the most ideal animal models to study IH.

TRPV5 (previously ECAC1), one member of calcium epithelium tunnel family, was cloned firstly in epithelial cells of rabbit collecting tubule[5]. Calbindin-D28k, one member of high affinity calcium binding protein family, is a transcellular Ca^{2+} transport protein which was discovered in chicken duodenum in recent years. NCX1 mainly distributes in basilemma of epithelial cells and discharges the endochylema Ca^{2+}. In human, TRPV5 and Calbindin-D28k mainly distribute in kidney, especially in distal convoluted tubule and collecting tubule. Both are 1,25$(OH)_2D_3$-dependent protein and play an important role in urine calcium reabsorption[6].

Fine-tuning of Ca^{2+} excretion is achieved by active transcellular reabsorption of Ca^{2+} in the distal convoluted tubule and connecting tubule. It involves apical Ca^{2+} entering through the epithelial Ca^{2+} channel TRPV5 (previously ECaC1), intracellular buffering and facilitated diffusion by the Ca^{2+}-binding protein Calbindin-D28k, and basolateral extrusion by the Na^+/Ca^{2+} exchanger (NCX1) and the plasma membrane Ca^{2+} ATPase (PMCA1b)[7]. In our study, we just investigated the implication of TRPV5, Calbindin-D28k and NCX1 in urine calcium reabsorption by detecting their expression respectively.

The expression of TRPV5 is regulated by many factors, and 1α,25$(OH)_2D_3$ is an important hormone of all factors. TRPV5 is 1,25$(OH)_2D_3$-dependent protein. 1,25 $(OH)_2D_3$ plays an basal role through binding its receptor VDR, so TRPV5 can be influenced by the number of VDR and functional status of body. Our previous study discovered that VDR was increased in intestinal tract and

kidney of GHS rats, moreover, half-life of VDR was prolonged. Because of these, we considered that overexpression of VDR in GHS rats resulted in pathopoiesis of IH[8, 9]. This paper discovered that the expression of TRPV5 was obviously decreased in kidney of GHS rats, consequently, more urine calcium was excreted and then hypercalciuria occurred. All of these illustrated the down-regulation of TRPV5 was a possible and main factor in IH. Of course, overexpression of VDR in GHS rats might result in the down-regulation of TRPV5 too, and further study is needed to understand the effect of VDR on TRPV5 by RNA interference.

However, the expression of Calbindin-D28k mRNA and protein in GHS rats was higher than in NC rats. Calbindin-D28k is also $1,25(OH)_2D_3$-dependent protein. Because GHS rats are characterized by VDR overexpression[8], as a result, the biological effect of VDR is magnified indirectly in normal $1,25(OH)_2D_3$, and the expression and synthesis of Calbindin-D28k protein are increased too. Nevertheless, considering the occurrence of IH, it is possible to cause IH when Calbindin-D28k is down-regulated. For this reason, we thought that Calbindin-D28k was not a major protein in IH. Of course, we did not deny the view that the stimulation of hypercalciuria induced the up-regulation of Calbindin-D28k as a compensatory adaptation[2, 10].

For NCX1, there was no significant difference between GHS rats and NC rats. Although urine calcium reabsorption is a consecutive transcellular process including TRPV5, Calbindin-D28k and NCX1, NCX1 might not play the same key role as above two proteins in urine calcium reabsorption.

Concerning IH, many researched concentrated on gastrointestinal tract. With further study, more and more attention must be paid in the role of kidney in IH. Elementary researches about TRPV5, Calbindin-D28k and NCX1 in this paper were carried out, including their possible effects on urine calcium reabsorption and IH. However, interaction among TRPV5, Calbindin-D28k, NCX1 and modulatory mechanism of $1,25(OH)_2D_3$ await further study. At the same tine, biological features of GHS rats and pathogenesis of urolithiasis need to be further investigated.

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