GENETICS

Investigation on Genetic Heterogeneity of α-Subunit of the Epithelial Sodium Channel in Rat Renal Cortex

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Genotypic variability of αENaC mRNA in adult rat kidney caused by deletion at the 3'-end of α-subunit DNA was studied by real-time PCR using specific probes with a fluorescent dye (TaqMan). It was found that mRNA deletion forms (a and b), products of alternative splicing of *aENaC* gene, are absent in the cortex of adult rat kidney.

Key Words: *kidney; epithelial sodium channel; intracellular sodium*

Epithelial sodium channel (ENaC) is the main transporter protein involved in regulated sodium reabsorption in the distal nephron segment. ENaC consists of α-, β-, and γ-subunits (2:1:1 ratio) surrounding the channel pore conducting sodium ions [3]. The causes of species- and tissue-specific variability of sodium channel properties attract much recent attention [5,7]. This attention can be explained by specific features of nucleotide sequences of DNA encoding sodium channel subunits. Deletions and untranslated sequences determine the existence of several mRNA forms for each subunit isoform, which modulates the properties of sodium channel [7,8]. Such diversity was not observed in cell cultures, because standard cDNA containing no untranslated sequences or deletions characteristic of native DNA are used for evaluation of the expression of ENaC subunits [9]. ENaC α-subunit contributes most to the heterogeneity of the channel properties. It is known that N-terminal of human *αENaC* contains a large untranslated DNA sequence determining a reading frame shift and production of sodium channel molecules of different activity [2,7-9]. On the other hand, deletion detected at the 3'-flanking region of the α-subunit DNA leads to alternative splicing [4] and their appearance of deletional α-subunit mRNA (a and b) forms of the epithelial sodium channel (*αENaC*) in addition to its wild type.

Here we studied the effect of deletion in the 3'-flanking region of α-subunit ENaC DNA on the ratio of wild and additional deletional mRNA forms (a and b) of this subunit in rat renal cortex.

MATERIALS AND METHODS

Renal cortex of adult male Wistar rats was used in the experiments. To assess genotypic diversity of αENaC mRNA, real-time PCR was performed using specific probes with fluorescent dye (TaqMan).

RNA from rat renal cortex was isolated using QIAGEN kit in accordance with manufacturer's instructions after preliminary freezing of the tissue.

For the design of oligonucleotide primers and probes, *αENaC* gene sequences were analyzed for wild-type (wt) and two deletion forms (a and b), products of alternative splicing of *αENaC* (Fig. 1) [4]. Primer se-

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lection criteria for reverse-transcription PCR (RT-PCR) analysis were the specific amplification of each form of *αENaC* (wt, a, and b) with appropriate primer pair: αENaC_upper_PCR/αENaC_wt_lower_PCR, αENaC_upper_PCR/αENaC_a_lower_PCR, αENaC_upper_PCR/αENaC_b_lower_PCR (Table 1).

Primer selection criteria for real-time PCR were specific amplification of each form of αENaC, wt, a, and b with a common primer pair were: αENaC_upper/ αENaC_lower (Table 1), and the length of accumulating amplicons (<250 b.p.). The probes were designed in such a way as to discriminate between the three

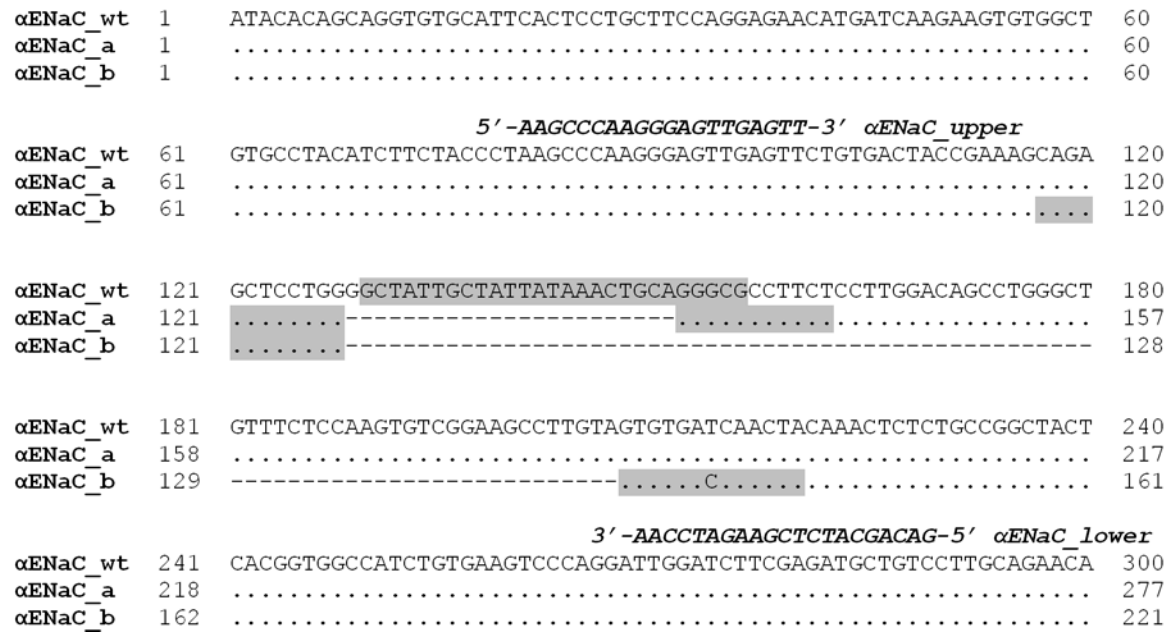

Fig. 1. Nucleotide sequence of wild-type *αENaC* gene (αENaC_wt) and its two alternatively spliced deletional forms (αENaC_a и αENaC_b). Identical nucleotides in the sequences relative to wild-type sequence (αENaC_wt) are indicated by dots while deletions are shown by dashes. Figures on the left and right of the nucleotide sequence indicate the position of nucleotides in the analyzed DNA segment. Sequences of oligonucleotide primers αENaC_upper and αENaC_lower are italicized. Gray letters show sequences complementary to hybridization probes αENaC_wt_probe, αENaC_a_probe, and αENaC_b_probe for fluorescence detection of wild-type αENaC DNA (αENaC_wt) and two deletional forms (αENaC_a and αENaC_b), respectively.

TABLE 1. Oligonucleotide Primers and Hybridization Probes Used in RT-PCR and Real-Time PCR

Primer/probe name	Nucleotide sequence of primer/probe
αENaC_upper	5’-AAGCCCAAGGGAGTTGAGTT-3’
αENaC_lower	5’-GACAGCATCTCGAAGATCCAA-3’
αENaC_wt_zond	5’-FAM-CGCCCTGCAGTTTATAATAGCAATAGC-BHQ1-3’
αENaC_a_zond	5’-FAM-AGAAGGCGCCCCCAGGAGC-BHQ1-3’
αENaC_b_zond	5’-FAM-TAGTTGGTCACACCCAGGAGCTCTG-BHQ1-3’
αENaC_upper_PCR	5’-CTGCTTCCAGGAGAACATGAT-3’
αENaC_wt_lower_PCR	5’-AGTTTATAATAGCAATAGCCCCAG-3’
αENaC_a_lower_PCR	5’-GAAGGCGCCCCCAGG-3’
αENaC_b_lower_PCR	5’-TTGTAGTTGGTCACACCCAGG-3’
GAPDH_upper	5’-AAGGCTGTGGGCAAGGTC-3’
GAPDH_lower	5’-AGGTTTCTCCAGGCGGC-3’
GAPDH_zond	5’-Cy5-CCATGCCAGTGAGCTTCCCGTT-BHQ3-3’
Random6	5’-NNNNNN-3’

forms of αENaC, wt, a, and b: αENaC_wt_primer, αENaC_a_primer, αENaC_b_primer (Table 1). To this end, alignment of DNA sequences for three *αENaC* gene splicing products (wt, a and b) was performed (Fig. 1). For deletions *αENaCa* and *αENaCb,* probes were designed for real-time PCR and oligonucleotide primers for RT-PCR enabling strictly specific identification of the three forms of *aENaC*: wt, a, and b. In RT-PCR, one of the primers was common to all three forms and the second primer in the pair was strictly specific for each form. In real-time PCR, the pair of primers was common to all three forms, and the probe was strictly specific for each variant of αENaC.

For quantitative analysis of the expression of three αENaC forms (wt, a, and b) by real-time PCR, primers and a probe for *GAPDH* gene with constant expression were calculated. Oligonucleotide primers and probes were synthesized by Syntol (Table 1).

Reverse transcription was performed in 20 μl of Sensiscript Reverse Transcriptase Kit (QIAGEN). The reaction mixture contained 1×RT Buffer, 500 μM dNTP, 10 mM Random6 primer (Table 1), 1.0 U Sensiscript Reverse Transcriptase, and 5 μl RNA solution. The mixture was incubated for 60 min at 37°C. The resultant cDNA was used in real-time RT-PCR analysis.

RT-PCR analysis was performed in 50 μl of One-Step RT-PCR Kit (QIAGEN). The reaction mixture contained 1×QIAGEN OneStep RT-PCR Buffer, 400 μM dNTP, 600 nM primers, 1.0 U QIAGEN OneStep RT-PCR Enzyme Mix, and 5 μl RNA solution. Amplification was carried out as follows: 30 min at 50°C; 15 min at 95°C; then 30 cycles: 30 sec at 94°C, 30 sec at 50°C, 1 min at 72°C; final stage: 10 min at 72°C.

Real-time PCR with the excision of 5'-terminal tags (TaqMan system) was performed in 25 μl using Applied Biosystems 7500 Real-Time PCR System. The reaction mixture contained 1×TaqMan Buffer A, 200 μM dNTP, 3 mM $MgCl_2$, 600 nM primers, 300 nM hybridization probe, 0.5 U AmpliTaq Gold DNA polymerase, and 1 μl cDNA solution. PCR with the measurement of fluorescence intensity at the annealing stage was carried out by the protocol: 2 min at 95°C, then 38 cycles: 15 sec at 95°C and 1 min at 58°C. All reactions were preformed in three repetitions.

Primers and a probe for *GAPDH* gene were used as the control in real-time PCR (Table 1).

RESULTS

In previous studies on rats, two deletion forms (a and b) of αENaC, alternative splicing products, were identified; in rat kidney αENaC_a predominated [4]. In order to quantify the contribution of these deletion forms to functional diversity of sodium channel properties in rat kidney, we assessed heterogeneity of *αENaC* mRNA determined by the presence of deletions in the 3'-end of the α-subunit DNA by real-time PCR.

Figure 2 shows the dependence of fluorescence intensity on the number real-time PCR cycle. A positive signal of specific expression was observed only for probe αENaC_wt. Probes for deletional forms αENaC_a and α ENaC_b mRNA yielded no fluorescent signal, which indicates the absence of alternative splicing products of αENaC in the renal cortex of adult rats. This result contradicted the data [4], so we carried out an additional check for the absence of deletional forms of αENaC RNA. We repeated the experiment many times with different concentration of the probe (from 100 to 900 nM). However, no positive fluorescence signal for probe αENaC_a or αENaC_b was obtained. At the same time, primers and probe for *GAPDH* gene in real-time PCR gave positive signal with cDNA of adult rat renal cortex (data not shown).

As an independent experiment, we performed RT-PCR-analysis using one common primer and specific primers for αENaC_wt, αENaC_a and αENaC_b relatively (Fig. 3). Indeed, only the pair of primers specific for αENaC_wt yielded a PCR product (length 123 b.p.). This experiment was also repeated several times with primer concentration from 200 to 900 nM. Primer annealing temperature was changed from 45°C to 55°C, however, no PCR product for primer pairs specific for αENaC_a or αENaC_b was obtained at this stage of our study.

These findings attest to the absence of deletional forms (a and b) of α*ENaC* gene in the renal cortex of adult Wistar rats. The mechanism providing mRNA

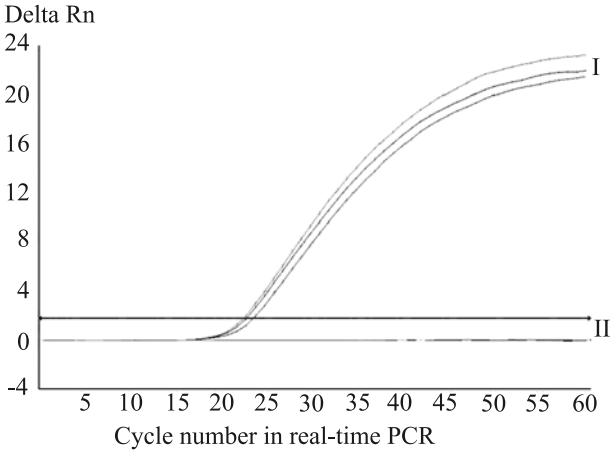

Fig. 2. Dependence of fluorescence intensity on the number of real-time PCR cycles. Data were obtained on Applied Biosystems 7500 Real-Time PCR System using oligonucleotide primers designed for the region of gene α*ENaC* and three probes: αENaC_wt, αENaC_a and αENaC_b. I, positive results for the probe αENaC_wt, II, negative results for the probes αENaC_a and αENaC_b, and NTC (No Template Control). Delta Rn, fluorescence signal intensity.

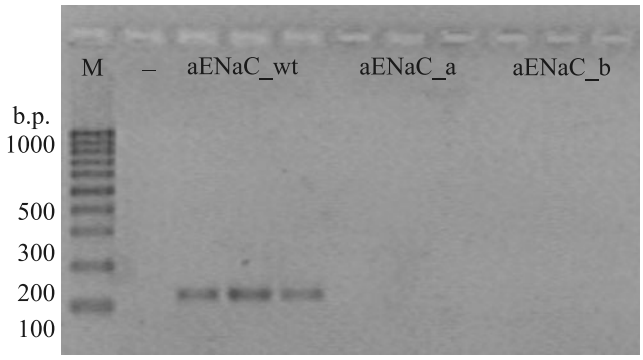

Fig. 3. Electrophoretic separation in 2% agarose gel of DNA fragments obtained after RT-PCR of rat renal cortex RNA using oligonucleotide primers for αENaC_wt, αENaC_a, and αENaC_b. Each reaction was conducted in 3 repetitions. "—": negative control, M: DNA marker.

heterogeneity in rat kidney probably includes finer regulation depending on the strain of experimental animals or conditions of their maintenance. For instance, changed balance between alternatively spliced forms αENaC-a and αENaC-b was revealed in salt-sensitive and salt-resistant Dahl rats after long-term high-salt diet [6]. In our experiment, we used adult male Wistar rats kept on a standard sodium diet. Unfortunately, the authors do not provide information on characteristics of animals and conditions of their maintenance [4]. Apart from species- and strain-specific features, genetic heterogeneity is affected by pathogenesis of hypertension, stress, gender, age [1,7,10]. Therefore, analysis of factors affecting the balance between deletional forms (a and b) of α*ENaC* gene seems quite relevant for better understanding of the regulatory mechanisms underlying genotypic diversity of mRNA caused by 3'-end deletion of αENaC DNA.

Thus, no alternatively spliced deletional forms (a and b) of *aENaC* mRNA were detected in renal cortex of male Wistar rats using RT-PCR and real-time PCR.

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