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Voltage-gated calcium channels in the human adrenal and primary aldosteronism

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ABSTRACT

Calcium channel blockers can efficiently be used in the treatment of primary aldosteronism (PA) related hypertension, but details on the localization of calcium channel (CC) in the human adrenal and its disorders, including PA, have remained unclear. Therefore, in this study we analyzed the known α subunits of L-, N- and T-type CCs in 74 adrenocortical aldosterone-producing adenomas (APA) and 16 cortisol-producing adenomas (CPA) using quantitative RT-PCR (qPCR). We also examined the status of L-(CaV1.2, CaV1.3), N-(CaV2.2) and T-(CaV3.2) CC subunits in five non-pathological adrenals (NA), five idiopathic hyperaldosteronism (IHA) cases, and 50 APA using immunohistochemistry. After qPCR evaluation, only CaV1.2, CaV1.3, CaV2.2, and CaV3.2 mRNA levels could be detected in APA and CPA. Among those, only CaV3.2 mRNA levels were significantly correlated with plasma aldosterone levels ($P = 0.0031$), *CYP11B2* expression levels ($P < 0.0001$) and the presence of *KCNJ5* mutations ($P = 0.0019$) in APA. The immunolocalization of CCs in NA and IHA was detected in the zona glomerulosa (ZG), with a predominance of CaV3.2 in APA. These findings suggest that different types of CC can be involved in calcium-related aldosterone biosynthesis.

1. Introduction

The zona glomerulosa (ZG) of the adrenal cortex produces and secretes aldosterone, which plays a pivotal role in maintaining electrolyte/water balance, and blood pressure homeostasis [1,2]. However, excessive plasma aldosterone levels cause not only arterial hypertension but also aggravate cardiac congestive failure, renal insufficiency and other disease processes [1–3].

A major cause of excessive plasma aldosterone levels is primary aldosteronism (PA). Recently, somatic mutations of the potassium ion (K^+) channel *KCNJ5* have been proposed to cause membrane depolarization with subsequent opening of voltage-gated calcium channel (CC) in aldosterone-producing adenomas (APA), one of the most frequent and important causes of PA [4]. The excessive influx of calcium (Ca^{2+}) in APA cells has been also reported to increase aldosterone synthesis in these tumors [4]. Calcium channel blockers (CCBs) have been considered effective in the treatment of PA patients as antihypertensive agents, in addition to aldosterone receptor blockers. CCBs have been reported to decrease not only the systemic blood pressure but also plasma aldosterone levels, possibly as a direct action on aldosterone producing adrenocortical cells [5–7]. However, the details on localization and functions of these channels in the human adrenal and its disorders, including PA, have not been reported, to the best of our knowledge. In particular, CCs have been previously detected in the ZG of bovine adrenal gland, but their localization in the human adrenal glands has remained unknown [8]. Therefore, in this study we evaluated the status of L-, N- and T-type CCs in non-pathological and hyperplastic human adrenal cortex, as well

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as APA; then, we compared the expression levels of those CCs mRNA to the status of *KCNJ5* mutations, the expression of steroidogenic enzymes involved in aldosterone biosynthesis, and clinical data of APA patients in order to further elucidate the details of CCs in neoplastic aldosterone overproduction.

2. Materials and methods

2.1. Human adrenals

The research protocols used in this study were approved by the Ethics Committee at Tohoku University Graduate School of Medicine (2011-543), Sendai, Japan.

For the quantitative RT-PCR analysis (qPCR), 74 APA and 16 cortisol-producing adenoma (CPA) frozen tissue specimens were also retrieved from tissue depository of Tohoku University Hospital, with clinical data retrieved from the respective clinical records.

For the immunohistochemical analysis, 5 non-pathological adrenal glands (NA), 5 idiopathic hyperaldosteronism (IHA) cases and 50 APA specimens were retrieved from surgical pathology files of Tohoku University Hospital (Sendai, Japan). NA samples were obtained from nephrectomy of patients with renal clear cell carcinoma and examined under light microscopy in order to confirm the absence of carcinoma metastasis, necrosis or other pathological findings.

2.2. RNA isolation and quantitative RT-PCR (qPCR)

RNA isolation with subsequent cDNA production and qPCR technique were performed as previously reported [9,10]. We evaluated and quantified the mRNA levels of all known α subunits of L-(CaV1.1, subunit α1S, *CACNA1S*; Cav1.2, subunit α1C, *CACNA1C*; CaV1.3, subunit α1D, *CACNA1D*; CaV1.4, subunit α1F, *CACNA1F*), N-(CaV2.2, subunit α1B, *CACNA1B*) and T-(CaV3.1, subunit α1G, *CACNA1G*; CaV3.2, subunit α1H, *CACNA1H*; CaV3.3, subunit α1I, *CACNA1I*) types of CC, as well as the steroidogenic enzymes CYP11B2 and CYP17. The primer sequences used in our study are specified in Table 1. The cDNA produced from a human brain specimen was used as a positive control in all CC qPCR experiments, while the cDNA of H295R cells was employed as a positive control for steroidogenic enzymes and *RPL13A*.

The relative gene expression was calculated as previously reported [10,11]. *RPL13A* was used as an endogenous control gene.

2.3. DNA sequencing and mutations in KCNJ5

APA cases ($n = 74$) were submitted to PCR using a *KCNJ5* primer (forward 5′-CGA CCA AGA GTG GAT TCC TT-′3, reverse 5′-AGG GTC TCC GCT CTC TTC TT-′3) as described by our group [11]. Analysis of the purified DNA was carried out with a Abi Prism 310 genetic analyser (Applied Biosystems, Foster City, CA, USA), and mutations at the G151R and L168R regions of the *KCNJ5* gene were analyzed as described [4].

2.4. Immunohistochemical staining and evaluation of immunoreactivity

Rabbit polyclonal antibodies against the α subunits of L-(CaV1.2; CaV1.3), N-(CaV2.2) and T-type (CaV3.2) CCs were purchased from Alomone Labs (Jerusalem, Israel). Immunostaining was performed using the streptavidin–biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan). Antigen retrieval was performed by heating the slides in a microwave for 15 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The dilutions of the primary antibodies used were: 1:200 for CaV1.2, CaV1.3 and CaV3.2, and 1:400 for CaV2.2. The antigen-antibody complex was visualized using 3,3′-diaminobenzidine solution [1 mM 3,3′-diaminobenzidine, 50 mM Tris-HCl buffer (pH 76), and 0006% H_2O_2] and counterstained with hematoxylin. Human brain tissues were used as positive control for these antibodies. Human spleen samples were used as negative controls.

After completely reviewing the slides, immunoreactivity was evaluated in each zone of the adrenal cortex as well as in tumor specimens by examining three different high-power fields and counting 500 parenchymal cells in each corresponding region. The ratio of positive cells was subsequently obtained in the immunostained areas of the NA and IHA and the APA cases. Immunoreactivity was assessed semiquantitatively using McCarty's H-scoring system, in which the percentage of immunopositive cells is multiplied by a number, 0–3, reflecting the intensity of their immunopositivities [12]. The relative immunointensity of specific immunoreactivity was characterized as not present (0), weak but detectable above control (1+), distinct (2+), and very strong (3+) [13]. The evaluation was independently and blindly carried out by two of the authors (S.J.A.F. and T.M.) and the mean values were used for analysis.

2.5. Statistical analysis

Data from the immunohistochemical analysis were evaluated in groups of three different categories (APA, IHA and NA) using Mann–Whitney multiple comparison tests with the significance level set to $\alpha = 0.05$. The Bonferroni inequality was used to correct the multiple comparisons, with $0.05/3 = 0.0167$ resulting in $P < 0.0167$ as the statistically significant value, as described [11].

qPCR data and clinical data of APA and CPA patients were evaluated either by regression analysis, or Mann-Whitney tests.

Table 1
RT-PCR primers based on human genome. L-type calcium channel α subunits: CaV1.1 (*CACNA1S*), CaV1.2 (*CACNA1C*), CaV1.3 (*CACNA1D*) and CaV1.4 (*CACNA1F*); N-type calcium channel α subunit: CaV2.2 (*CACNA1B*); T-type calcium channel α subunits: CaV3.1 (*CACNA1G*), CaV3.2 (*CACNA1H*) and CaV3.3 (*CACNA1I*).

Gene/primer	Forward	Reverse
CACNA1B	5′- TGACTGTGTGGTGAACTCCCTGAAGAA-′3	5′-ACCGCAATGACGGCAAATATGAACATG-‘3
CACNA1C	5′-CCTTTCTGGTTTAGCTGTGGGAAGATCT-′3	5′-AATGCAAAGAGTTACTGATTCCCGTTTCAG-′3
CACNA1D	5′-AGGAGGACCTGGCGGATGAAATGATAT-′3	5′-ACTCCCAACTAGTGCCTAAACTTTCCTAAC-′3
CACNA1F	5′-ACGGTGGAGATGCTTCTCAAATTGTACG-′3	5′-GGTGACCTTAAAGATCCTGAGGAGGC-′3
CACNA1G	5′-GAAGCAGCTTAGAGTTGGACACGGA-′3	5′-GCAGCTGACGGCGATAGAGTGTC-′3
CACNA1H	5′-CACACCGATTCCTTGGAAGGGAAGATT-′3	5′-GCTGTCCGAAGGTATGCTTACGAGTG-′3
CACNA1I	5′-AGGGAGTGACTTTCATGAAGTCAGTTTGAA -′3	5′-GCTGAAGTACAGCATCTGCAAACTCGT-′3
CACNA1S	5′-CTGCTGTACAAGGCCATAGACTCCAAT-′3	5′-TCACAGTTCTTGTACTCAGTCTCTCCCT-′3
CYP11B2	5′-TCCAGGTGTGTTCAGTAGTTCC-′3	5′-GAAGCCATCTCTGAGGTCTGTG-′3
CYP17	5′-TGAGTTTGCTGTGGACAAGG-′3	5′-TCCGAAGGGCAAATAGCTTA-′3
RPL13A	5′-CCTGGAGGAGAAGAGGAAAG-′3	5′-TTGAGGACCTCTGTGTATTT-′3