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Expression and localization of calmodulin-related proteins in brain, heart and kidney from spontaneously hypertensive rats

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Blood pressure is regulated not only by peripheral arterial resistance, but also by heart, kidney, and central nervous system. We have previously demonstrated that expression level of calmodulin-related proteins including eukaryotic elongation factor 2 kinase (eEF2K), death-associated protein kinase (DAPK)3, and histone deacetylase (HDAC)4 was specifically elevated in mesenteric artery from spontaneously hypertensive rats (SHR), which partly contributes to the development of hypertension via vascular inflammation and structural remodeling. We tested the hypothesis whether expression and localization of eEF2K, DAPK3, and HDAC4 are altered in brain, heart, and kidney from SHR. After brain, left ventricles (LV), and kidney were isolated from 12-week-old male Wistar Kyoto rats (WKY) and SHR, Western blotting and histological analysis were performed. In brain tissue, protein expression of eEF2K and HDAC4 was abundant, whereas DAPK3 protein was less. HDAC4 protein expression in SHR brain was significantly higher than that in WKY brain. In LV, protein expression of eEF2K was relatively higher than DAPK3 or HDAC4, and it was significantly higher in SHR than WKY. In kidney tissue, protein expression of DAPK3 was the highest and seemed to be localized specifically to renal tubule. The present results indicate that the increased HDAC4 in brain and increased eEF2K in LV might be at least in part related to the development of hypertension.

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1. Introduction

Calmodulin (CaM), a calcium binding protein, regulates various cellular functions such as smooth muscular contraction [1], cell cycle [2], and metabolism [3] via CaM-related proteins. We have previously demonstrated that protein expression of CaM-related proteins including eukaryotic elongation factor 2 (eEF2) kinase (eEF2K), death-associated protein kinase (DAPK)3 and histone deacetylase (HDAC)4 was specifically elevated in superior mesenteric artery from spontaneously hypertensive rats (SHR) compared with normotensive Wistar Kyoto rats (WKY) [4]. Furthermore, we revealed that these molecules mediate the elevation of blood pressure in SHR at least in part through enhancing peripheral arterial resistance via promoting tumor necrosis factor-α-induced reactive oxygen species-dependent inflammatory responses [5–7] and platelet-derived growth factor-BB-induced migration and proliferation [8–10] in mesenteric arterial smooth muscle cells.

Systemic blood pressure is mainly regulated by peripheral arterial resistance and blood volume, which are affected not only by blood vessel but also by several tissues including heart, kidney and brain. It is recently recognized that Ca^{2+}/CaM-dependent protein kinase (CaMK)II, one of the CaM-related proteins, plays a crucial role in the pathogenesis of certain cardiovascular diseases through regulating the functions of these tissues. In hypertrophied myocardium from SHR, for example, expression [11] and activity of CaMKII [12] were increased. CaMKII also mediated angiotensin II-induced podocytopathy and albuminuria via cyclic adenosine monophosphate response element-binding protein/Wnt/β-catenin signaling pathway [13]. In addition, CaMKII modulated neuronal cell death after cerebral ischemia [14]. We thus hypothesized that other CaM-related proteins including eEF2K, DAPK3 and HDAC4 may mediate the development of essential hypertension thorough

Abbreviations: CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats; eEF2, eukaryotic elongation factor 2; eEF2K, eEF2 kinase; DAPK, death-associated protein kinase; HDAC, histone deacetylase; SBP, systolic blood pressure; LV, left ventricles; BW, body weight; RVLM, rostral ventrolateral medulla; ERK, extracellularly regulated kinase.

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regulating the functions of heart, kidney and brain. To test the hypothesis, we explored the changes in expression level and localization of these molecules in heart, kidney and brain from SHR with developed hypertension. We for the first time found that protein expression of HDAC4 in brain and that of eEF2K in left ventricles (LV) were specifically increased in SHR.

2. Material and methods

2.1. Animals

Animal care and treatment were conducted in accordance with the institutional guidelines of The Kitasato University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal study was approved by the ethical committee of School of Veterinary Medicine, The Kitasato University. After systolic blood pressure (SBP) of 12-week old male SHR (Hoshino Laboratory Animals, Inc., Ibaraki, Japan) and age-matched male WKY was measured by a tail-cuff method as described previously [7], the rats were euthanized by exsanguination under a deep urethane (1.5 g/kg, i.p.) anesthesia. Brain, LV and kidney tissues were then isolated and weighed. These tissues were immediately frozen in −80 °C for protein extraction and also fixed in 10% neutral buffered formalin for histological and immunohistochemical examinations.

2.2. Western blot analysis

Western blotting was performed as described previously [15]. Protein lysates were obtained by homogenizing tissue samples with Triton-based lysis buffer. Protein concentration was measured using a bicinchoninic acid method (Pierce, Rockford, IL, USA). Equal amounts of proteins (10 μg) were separated by SDS-PAGE (10%) and transferred to a nitrocellulose membrane (Pall, Ann Arbor, MI, USA). After blocked with 0.5% skim milk, membranes were incubated with the following first antibodies (1:500 dilution): total eEF2K, total DAPK3 and total HDAC4 at 4 °C overnight and visualized using horseradish peroxidase-conjugated second antibody (1:10000 dilution, 45 min at room temperature) and the EZ-ECL system (Biological industries, Kibbutz Beit Haemek, Israel). Equal loading of protein was verified by measuring β-actin expression. The results were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

2.3. Histology and immunohistochemistry

The isolated LV and left kidney were fixed in 10% neutral buffered formalin. The thin paraffin sections (4 μm) of LV were made and stained with hematoxylin and eosin as described previously [15]. For immunohistochemistry, the deparaffinized sections of LV and kidney were firstly heated using a microwave for antigen retrieval. Endogenous peroxidase activity was blocked by incubating in Dako REAL peroxidase-blocking solution (Dako, Glostrup, Denmark) for 15 min. The sections were blocked for 60 min with 5% normal goat serum and subsequently incubated with first antibody (1:250 dilution) against eEF2K or DAPK3 at 4 °C overnight. After washed by Tris buffer, the sections were incubated in biotinylated link (Dako) for 10 min and next in streptavidin-HRP (Dako) for 10 min at room temperature, and were visualized by a liquid DAB + substrate chromogen system (Dako). The images were obtained using a light microscope (BX-51, Olympus, Tokyo, Japan). For evaluating cardiac hypertrophy, cross-sectional area (μm^2) of three cardiomyocytes in three fields from each LV section was calculated using Image J software (NIH, Bethesda, MD, USA). For comparison of eEF2K expression level between LV of WKY and SHR, ratio of eEF2K-positive area in three fields from each LV section was calculated using Image J software.

2.4. Materials

Antibody sources were as followings: total-eEF2K (No. GTX107879) (Gene Tex, Irvine, CA, USA); total DAPK3 (No. GTX102404) (Gene Tex); total HDAC4 (No. E021141) (Eno Gene, New York, NY, USA); β-actin (No. 21338) (Signalway Antibody, College Park, MD, USA).

2.5. Statistics

Data were shown as mean ± SEM. Comparison between six groups was performed by one-way ANOVA followed by Bonferroni's test. Comparison between two groups was performed by unpaired student's *t-test*. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Physical parameter and tissue weight

Body weight (BW) of SHR was significantly lower than that of WKY (281.0 ± 3.8 g vs. 260.4 ± 5.5 g, n = 7, $p < 0.01$, Table 1). SBP (114.6 ± 5.7 mmHg vs. 179.3 ± 2.8 mmHg, n = 7, $p < 0.01$, Table 1) and LV weight to BW ratio (LV/BW; 2.62 ± 0.08 mg/g vs. 3.11 ± 0.06 mg/g, n = 7, $p < 0.01$, Table 1) in SHR were significantly higher than those in WKY. Left kidney or right kidney to BW ratio was not different (n = 7, Table 1).

3.2. Changes in protein expression level of eEF2K, DAPK3, and HDAC4 in brain, LV, and kidney from SHR

We first compared the protein expression level of eEF2K, DAPK3, and HDAC4 in brain, LV, and kidney, which can potentially affect blood pressure. In brain tissue of WKY, protein expression level of eEF2K and HDAC4 was similar, whereas DAPK3 protein was less (n = 5, Fig. 1AB). HDAC4 expression in SHR brain was significantly higher than that in WKY brain (n = 5, $p < 0.01$ vs. HDAC4 in WKY, Fig. 1AB). In LV tissue of WKY, protein expression of eEF2K was relatively higher than that of DAPK3 or HDAC4 (n = 7, Fig. 1AC). eEF2K expression in SHR LV was significantly higher than that in WKY LV (n = 7, $p < 0.05$ vs. eEF2K in WKY, Fig. 1AC). In left and right kidney tissue of WKY, expression of DAPK3 protein was higher than that of eEF2K or HDAC4 protein (n = 7, Fig. 1A and DE). There is no significant difference in expression of each protein between WKY and SHR (n = 7, Fig. 1A and DE).

3.3. Cardiac hypertrophy and expression and localization of eEF2K protein in LV

We next examined the LV hypertrophy and the expression and localization of eEF2K protein in LV by histological examinations. Cross-sectional area of LV cardiomyocyte in SHR was significantly higher than that in WKY (266.6 μm^2 vs. 400.7 μm^2, n = 5, $p < 0.01$, Fig. 2A-a, b, and B). We also confirmed that the ratio of eEF2K-positive area in LV cardiomyocyte from SHR was significantly higher than that from WKY (1.84-fold relative to WKY, n = 5, $p < 0.05$, Fig. 2A-c, d, and C). eEF2K expression seemed to be primary localized to cardiomyocytes.

3.4. Expression and localization of DAPK3 protein in kidney

In the Western blot analysis, it was shown that the expression of DAPK3 protein was higher than that of eEF2K or HDAC4 in the

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