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Exploring calmodulin-related proteins, which mediate development of hypertension, in vascular tissues of spontaneous hypertensive rats

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ABSTRACT

Calmodulin (CaM) is associated with a variety of cell functions including inflammation, apoptosis, and muscular contraction. It is recently clarified that some CaM-related proteins are responsible for cardiovascular diseases. We therefore explored CaM-related proteins that mediate hypertensive vascular diseases. Expression levels of six CaM-related proteins with almost unknown function in blood vessels were examined in aorta and mesenteric artery from spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) by Western blotting. In aorta from SHR, eukaryotic elongation factor (eEF)2 kinase (eEF2K) and death-associated protein kinase (DAPK)3 protein increased compared with WKY, while Ca^{2+}/CaM-dependent protein kinase IIδ, histone deacetylases (HDAC)4 and HDAC5 protein decreased. In mesenteric artery from SHR, eEF2K, HDAC4 and DAPK3 protein increased compared with WKY, while HDAC5 decreased. Our findings demonstrate that expression levels of several CaM-related proteins are changed in vascular tissues of SHR and suggest that CaM-related proteins might be at least in part related to the pathogenesis of hypertensive vascular diseases.

1. Introduction

Calmodulin (CaM) is a multifunctional regulator, which primarily works as a sensor of an intracellular Ca^{2+}. It is also known that CaM is involved in pathogenesis of heart failure. For example, overexpression of CaM promotes cardiac hypertrophy through activation of Ca^{2+}/CaM-dependent protein kinase (CaMK)II in transgenic mice [1]. Activation of CaMKII is regulated by the binding of CaM, and it undergoes autophosphorylation at Thr286 in the presence of CaM [2]. CaMKII causes hypertrophy via activation of transcriptional factor histone deacetylase (HDAC) and myocyte-specific enhancer factor (MEF)2 pathways in cardiomyocytes [3]. In addition, it was demonstrated that a CaMKII inhibitor, KN93 suppressed the angiotensin (Ang) II-induced hypertrophy of vascular smooth muscle cells [4].

Reactive oxygen species (ROS) generation in vascular endothelial and smooth muscle cells is critical for development of atherosclerosis and hypertensive diseases. It was shown that CaM/CaMKII is associated with regulation of ROS generations. For example, CaM increases mitochondrial ROS generation in rat ventricular myocytes [5]. Conversely, in catecholaminergic neurons, Ang II-induced mitochondrial O^{2-} production regulates activation of CaMKII [6]. Furthermore, in a recent study, we have demonstrated that CV-159, a Ca^{2+}/CaM antagonist, inhibits vascular smooth muscle inflammatory responses by suppressing ROS generation and CaM/CaMKII activation [7]. These results suggest that inhibition of CaM/CaMKII might have impacts on cardiovascular diseases including atherosclerosis and hypertension.

It is known that CaM regulates various proteins other than CaMKII. Recently, it was reported that these CaM-related proteins are also involved in the mechanisms of heart failure or cerebral infarction. For example, (1) activation of eukaryotic elongation factor (eEF)2 kinase (eEF2K) (known as CaMKIII) contributed to protection of cardiomyocytes against hypoxic injuries [8]. (2) an inhibition of CaM-related protein kinase, death-associated protein kinase (DAPK) improved hypoxic ischemia-induced acute brain disorders in rats [9]. However, it remains to be fully clarified how the CaM-related proteins modulate such diseases. Moreover, it is unknown whether or not other CaM-related proteins are responsible for cardiovascular diseases. Thus, we compared expression levels of six CaM-related proteins in aorta and mesenteric artery from SHR and WKY. Here, we for the first time demonstrate that expressions of these proteins are actually changed in SHR compared with WKY.

2. Materials and methods

2.1. Materials

Antibody sources were as follows: CaMKIIδ, eEF2K, CaM serine kinase (CASK) and DAPK3 (Gene Tex, Irvine, CA, USA); HDAC4

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Table 1
Body weight, heart rate (HR), systolic blood pressure (SBP), diastolic BP (DBP), and mean BP (MBP) in WKY and SHR.

	WKY (n = 6)	SHR (n = 6)
Weight (g)	305.7 ± 7.8	289.7 ± 4.7
HR (bpm)	320.6 ± 11.9	348.5 ± 26.3
SBP (mmHg)	118.9 ± 2.1	162.2 ± 5.5**
DBP (mmHg)	84.0 ± 2.8	119.4 ± 3.7**
MBP (mmHg)	95.5 ± 2.1	133.7 ± 4.1**

** $P < 0.01$ vs. WKY.

and HDAC5 (Eno Gene, Nanjing, China); total actin (Sigma Aldrich, St. Louis, MO, USA).

2.2. Blood pressure measurement

Measurements of systolic blood pressure (SBP), diastolic BP (DBP) and mean arterial BP (MBP) in conscious spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) were performed using a tail-cuff system (Softron, Tokyo, Japan).

2.3. Tissue preparation

SHR (0.28–0.31 kg: 13–15-week old; Hoshino Laboratory Animals, Inc., Ibaragi, Japan) and age-matched WKY (0.29–0.34 kg: 13–15-week old) were anesthetized with urethane (1.5 g/kg, i.p.) and euthanized by an exsanguination. The thoracic aorta and superior mesenteric artery were isolated as described previously[10,11]. After the adipose and connective tissues were removed, protein was extracted as described below. Animal care and treatment were conducted in conformity with institutional guidelines of The Kitasato University.

2.4. Western blotting

Western blotting was performed as described previously [7,12]. Protein was extracted by homogenizing aorta and mesenteric artery with Triton-based lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM NA_3VO_4, 1 μg/ml leupeptin, and 0.1% protease inhibitor cocktail; Nacalai Tesque, Kyoto, Japan). Protein concentration was measured by using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Equal amount of proteins (10–15 μg) were separated by SDS–PAGE (7.5%), and transferred to a nitrocellulose membrane (Pall, Ann Arbor, MI, USA). After a blocking with 0.5% skim milk, membranes were incubated with first antibodies at 4 °C over night, and then visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 h) and the EZ-ECL system (Biological industries, Kibbutz Beit Hesmek, Israel). Equal protein loading was confirmed by measuring total actin expression. The resulting autoradiograms were analyzed by using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

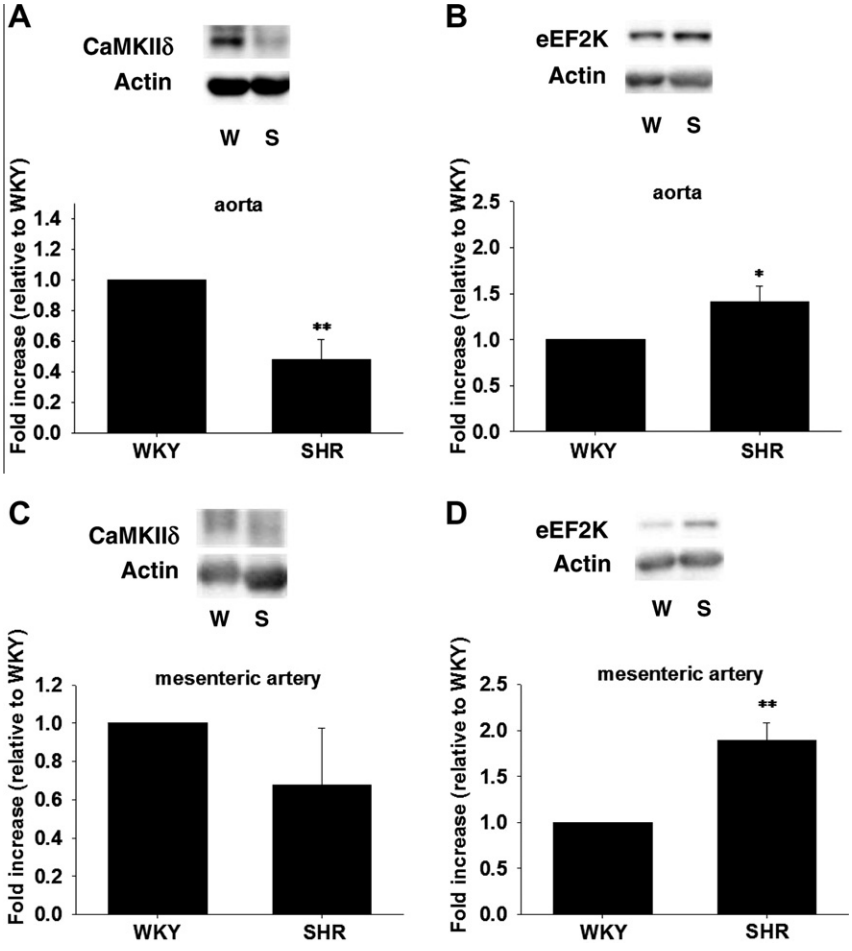

Fig. 1. Protein expression levels of CaMKIIδ and eEF2K in aorta (A, B) and mesenteric artery (C, D) from SHR and WKY. Protein expression of CaMKIIδ (A; n = 4, C; n = 4) and eEF2K (B; n = 5, D; n = 4) was determined by Western blotting. Equal protein loading was confirmed using total actin antibody. Expression level is shown as fold increase relative to WKY.*: Significantly different from WKY with $P < 0.05$, **: Significantly different from WKY with $P < 0.01$.

2.5. Statistical analysis

Results are expressed as mean ± S.E.M. Statistical evaluation of the data was performed by unpaired Student's *t*-test. Results were considered significant when P was less than 0.05.

3. Results

3.1. Parameters of experimental animals

SBP, DBP and MBP in SHR are significantly higher than those in WKY (Table. 1, $P < 0.01$).

3.2. Expression levels of CaMK proteins in aorta and mesenteric artery from SHR and WKY

We first compared expression levels of several CaMK proteins in aorta and mesenteric artery between SHR and WKY. In aorta from SHR, CaMKIIδ protein significantly decreased compared with WKY (Fig. 1A, 48.0 ± 12.9% relative to WKY, $P < 0.01$, $n = 4$), while eEF2K (also known as CaMKIII) significantly increased (Fig. 1B, 141.4 ± 16.9% relative to WKY, $P < 0.05$, $n = 5$). In mesenteric artery from SHR, CaMKIIδ protein did not change compared with WKY (Fig. 1C, $n = 4$), while eEF2K significantly increased (Fig. 1D, 188.8 ± 19.8% relative to WKY, $P < 0.01$, $n = 4$).

3.3. Expression levels of HDAC proteins in aorta and mesenteric artery from SHR and WKY

We next compared protein expression levels of CaMKII-related transcriptional factor, HDACs in aorta and mesenteric artery between SHR and WKY. In aorta from SHR, both HDAC4 and HDAC5 protein significantly decreased compared with WKY (Fig. 2A, 54.6 ± 5.1% relative to WKY, $P < 0.01$, $n = 5$; Fig. 2B, 62.2 ± 9.3% relative to WKY, $P < 0.01$, $n = 4$). In mesenteric artery from SHR, HDAC4 protein significantly increased compared with WKY (Fig. 2C, 178.0 ± 18.9% relative to WKY, $P < 0.01$, $n = 5$), while HDAC5 significantly decreased (Fig. 2D, 46.5 ± 11.5% relative to WKY, $P < 0.01$, $n = 4$).

3.4. Expression levels of CaM-related protein kinases in aorta and mesenteric artery from SHR and WKY

It was next analyzed protein expression levels of other CaM-related family protein kinases in aorta and mesenteric artery from SHR compared with WKY. In aorta and mesenteric artery from SHR, CASK protein did not change compared with WKY (Fig. 3A, $n = 5$; Fig. 3C, $n = 5$), while DAPK3 protein significantly increased (Fig. 3B, 169.4 ± 17.3% relative to WKY, $P < 0.01$, $n = 4$; Fig. 3D, 240.8 ± 49.8% relative to WKY, $P < 0.05$, $n = 5$).

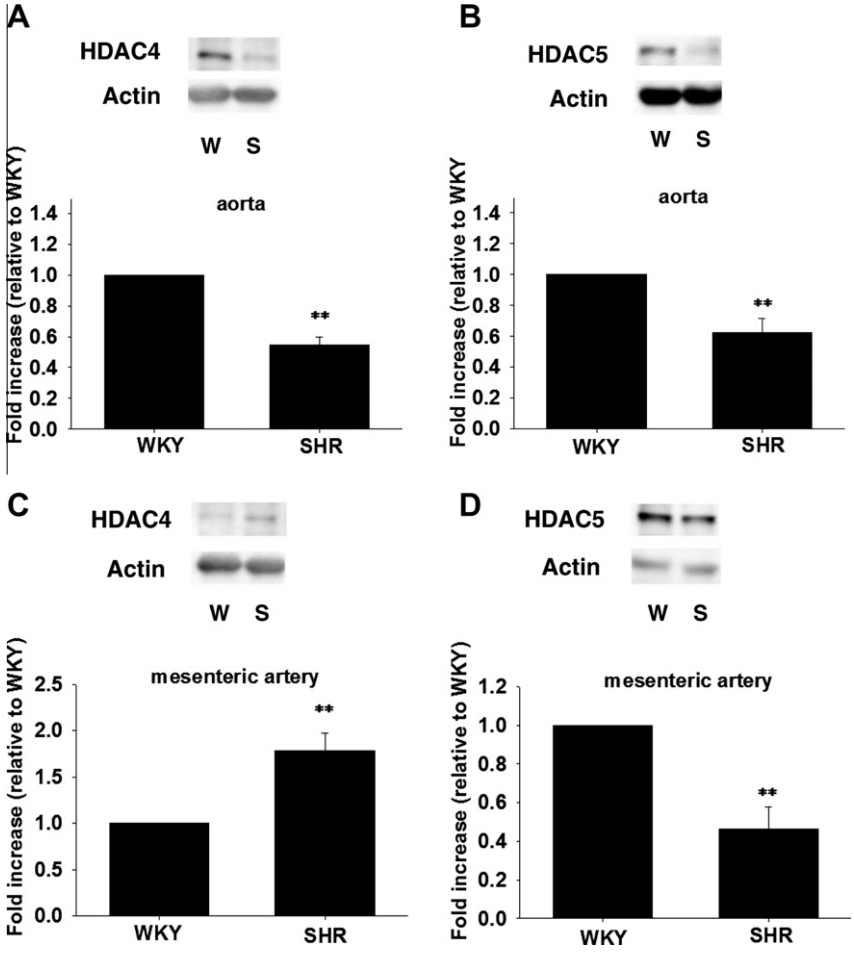

Fig. 2. Protein expression levels of HDAC4 and HDAC5 in aorta (A, B) and mesenteric artery (C, D) from SHR and WKY. Protein expression of HDAC4 (A; $n = 5$, C; $n = 5$) and HDAC5 (B; $n = 4$, D; $n = 4$) was determined by Western blotting. Equal protein loading was confirmed using total actin antibody. Expression level is shown as fold increase relative to WKY. **: Significantly different from WKY with $P < 0.01$.

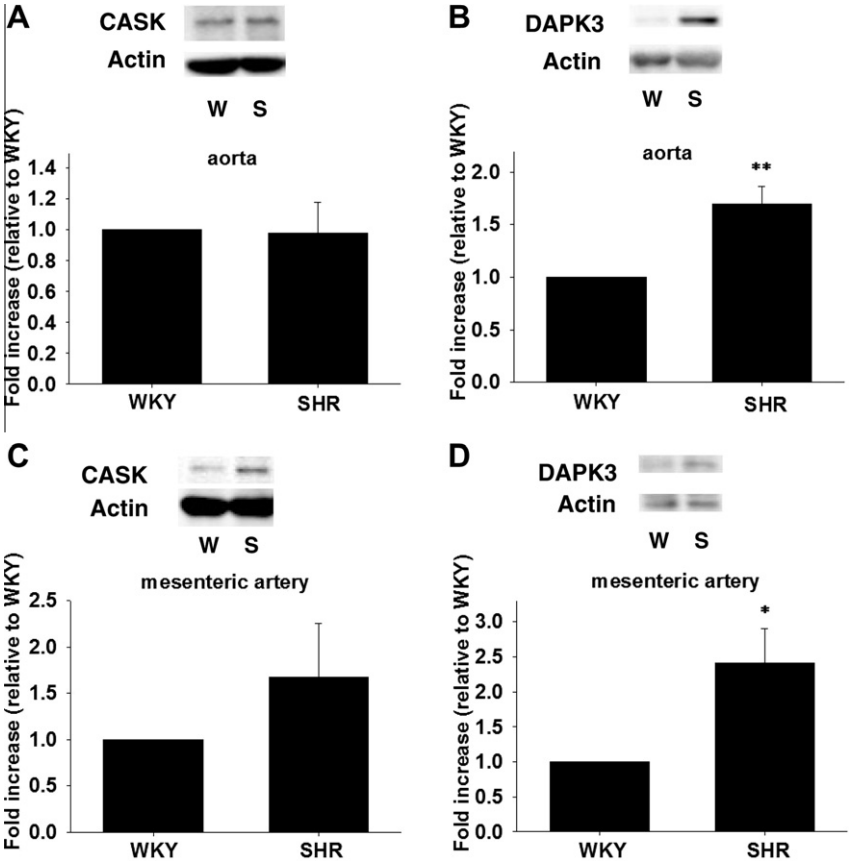

Fig. 3. Protein expression levels of CASK and DAPK3 in aorta (A, B) and mesenteric (C, D) artery from SHR and WKY. Protein expression of CASK (A; $n = 5$, C; $n = 5$) and DAPK3 (B; $n = 4$, D; $n = 5$) was determined by Western blotting. Equal protein loading was confirmed using total actin antibody. Expression level is shown as fold increase relative to WKY.∗: Significantly different from WKY with $P < 0.05$, ∗∗: Significantly different from WKY with $P < 0.01$.

4. Discussion

In the present study, we compared expression levels of several CaM-related proteins in vascular tissues of SHR and WKY. The major findings of the present study are that the expressions of several CaM-related proteins are confirmed in aorta and mesenteric artery and that their expression levels are different between SHR and WKY. It is suggested that the CaM-related proteins might be at least in part related to the pathogenesis of hypertensive vascular diseases, although causal links were not determined in this study.

CaMKII is a ubiquitous mediator of Ca^{2+} signaling, and autophosphorylated at Thr286 upon binding to Ca^{2+}/CaM, which in turn activates numerous proteins including myosin light chain kinase and mitogen-activated protein kinase [13]. Recently, it was reported that CaMKII controls cardiovascular diseases, including hypertrophic and hyperresponsive diseases. For example, (1) in cardiomyocyte, CaMKII caused hypertrophy via activation of the HDAC and MEF2 [3], (2) a CaMKII inhibitor inhibited the Ang II-induced vascular smooth muscle cell hypertrophy [4]. (3) The increased vascular responsiveness to endothelin-1 and Ang II in isolated carotid arteries from streptozotocin-treated rats was reversed by a CaMKII inhibitor [14]. In addition, we have recently demonstrated that CV-159, a Ca^{2+}/CaM antagonist, inhibited the TNF-induced expression of vascular cell adhesion molecule-1 via suppressing the activation of Akt and NF-κB in vascular smooth muscle cells [7]. It was found in the study that CV-159 prevents NF-κB phosphorylation by inhibiting ROS, whereas CV-159 prevents Akt phosphorylation by inhibiting both ROS and CaM/CaMKII. These results suggest that in addition to hypertrophy and hypercontractility, CaM/CaMKII regulates inflammatory responses of vascular smooth muscle, which are responsible for atherosclerosis and/or hypertensive diseases. We therefore hypothesized that other CaM-related proteins with almost unknown function in vascular tissues might also be involved in the pathogenesis of cardiovascular diseases including hypertensive vascular diseases.

Accordingly, in the present study we compared expression levels of the following six CaM-related proteins in vascular tissues of SHR and WKY. (1) CaMKII has four isoforms termed α, β, γ and δ [15]. It was reported that the expression of CaMKIIδ increases in failing human hearts [16]. Recent study has demonstrated that in mouse dilated cardiomyopathy, CaMKIIδ causes heart failure by increasing the number of apoptotic cardiomyocytes and upregulation of p53 protein [17]. (2) eEF2K is known as CaMKIII, which phosphorylates eEF2 at Thr56. It was reported that eEF2 regulates protein synthesis by affecting the elongation stage of translation [18,19]. It was demonstrated that activation of eEF2K by 5′-AMP-activated protein kinase contributes to protection of heart against hypoxic injuries [8]. (3) HDACs play a role on the transcriptional regulations during the cellular responses [20]. In cardiomyocytes, it was shown that CaMKIIδ is the specific HDAC4 kinase [21]. It was also demonstrated in cardiomyocytes that HDAC4 and HDAC5 are exported to the cytoplasm in response to phenylephrine [3]. (4) CASK, a membrane-associated guanylate kinase protein, has been shown to play a role on the development and function of brain in both rodents and humans [22,23]. (5) DAPK is a Ca^{2+}/CaM-regulated serine/threonine kinase that mediates cell death [24]. It was reported that DAPK promotes apoptosis and autophagy [24,25]. In the present study, our data showed that eEF2K and DAPK protein in aorta from SHR increases compared with WKY, while in mesenteric artery eEF2K, HDAC4 and DAPK3 protein

increases. We further showed that HDAC4, HDAC5 and CaMKIIδ protein in aorta from SHR decreases compared with WKY, while in mesenteric artery HDAC5 protein decreases. We suppose that these CaM-related proteins might at least in part play a role on the pathogenesis of hypertensive vascular diseases.

In summary, we for the first time demonstrated that in vascular tissues from SHR, expressions of several CaM-related proteins are changed compared with WKY. However, it remained to be clarified how each CaM-related protein might affect vascular pathophysiology. Further studies are necessary to clarify it.

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