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유전적 고혈압 발병에 대한 Calcineurin 및 PKB/Akt의 연관성

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Involvement of calcineurin and PKB/Akt in development of hereditary hypertension

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Abstract : Severe hypertension (>180 mmHg) develops in spontaneously hypertensive rats (SHR) after 12 wk-old; however, it is not clear whether what kinds of molecular mechanism leads to altered cardiac performance following developmental stages in SHR. Also, although the effect of calcineurin (Cn) to promote cardiomyocyte hypertrophy *in vivo* and *in vitro* is established, its overall necessity as a hypertrophic mediator is currently an area of ongoing debate. Thus, we have examined i) body weight and blood pressure, ii) differences of expression and distribution of signaling molecules such as Cn, protein kinase B/Akt (PKB/Akt), and extracellular signal-regulated kinase (ERK) between SHR and their age-matched control Wistar-Kyoto (WKY) rats following developmental stages. In 16 wk-old SHR compared with WKY, 2-dimentional echocardiography showed cardiac enlargement and hypertrophy of left ventricle, significantly. Taken together, we suggest that Cn is associated with hereditary cardiac hypertrophy, the process being related to the molecular signaling mechanisms involving PKB/Akt and ERK.

Key words : spontaneously hypertensive rat, hypertension, hypertrophy, calcineurin, PKB/Akt, ERK

Introduction

There are two main types of hypertension [21]. Primary or essential hypertension accounts for over 90% of all patients and the secondary makes up the rest. As the name implies, the essential hypertension has no obvious known cause. The heart is one of the main target organs in hypertension. Changes in myocyte expression of both contractile and noncontractile proteins and increased interstitial fibrosis are typically found during the natural course of hypertensive heart disease and result in progressive pathological hypertrophy, which may lead to heart failure (HF) [2, 4, 17, 22]. The SHR is a well-established model of genetic hypertension, which in certain ways resembles hypertension in humans [3]. However, it is not clear how hypertrophy develops following developmental stages in spontaneously hypertensive rat (SHR). So far, several studies involving different experimental models of pressure-induced hypertrophy have demonstrated that systolic performance

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is either normal, increased, or decreased before the development of HF.

Bing *et al*. [2] also demonstrated that cardiac function was impaired with aging in SHR, highlighting the importance of both myocytes and the interstitium in the transition to HF. Pfeffer *et al*. [20] found cardiac output to be increased in young SHR compared with Wistar-Kyoto (WKY) rats, whereas Kobayashi *et al*. [13] found increased contractility in myocytes isolated from older SHR. But it is still remained as known mechanism that what kinds of mechanism are involved in development of hypertrophy and eventually hypertension following developmental stages in SHR.

After various pathologic stresses and in response to increased demands for cardiac work, the heart adapts through compensatory hypertrophy of myocytes, which is characterized by an increase in the size of myocytes (cardiac hypertrophy), and the expression of contractile and other proteins were normally expressed only during fetal development [14, 15]. While the hypertrophic response is initially a compensatory mechanism that augments cardiac output, sustained hypertrophy can lead to dilated cardiomyopathy, heart failure and sudden death. Despite the diverse stimuli that lead to cardiac hypertrophy, there is a prototypical final molecular response of cardiomyocytes to hypertrophic signals that involves an increase in cell size, cell number and protein synthesis, enhanced sarcomeric organization, up-regulation of fetal cardiac genes, and induction of immediate-early genes, such as c-Jun, c-fos and c-myc [16]. Cardiac hypertrophy is induced by a variety of factors, such as vasoactive peptides, growth factors, cytokines, and hormones [7]. Especially, catecholamines (Phenylephrine, Isoproterenol) also play pivotal roles in cellular growth [6]. There are main hypertrophic signaling pathway has been described which involves activation of the Ca^{2+}/Calmodulin-dependent serine-threonine phosphatase calcineurin (Cn) [5]. Persistent stimulation of cardiac cells by catecholamines, for example, has been known as a prime example of calcineurin-induced cardiac hypertrophy [1].

Agonist (Endothelin-1, Angiotensin-II) activates are G-protein-coupled receptors (GPCR), to activate PI3-kinase, which activate PKB/Akt and Ca^{2+} release from intracellular. Thus, initiating calcium-activated signaling pathway [16], then Ca^{2+} binds with calmodulin, which activated Cn. Thus, the elevation of free Ca^{2+} concentration in cytosol, is increased binding with calmodulin, resulting in Cn activation [8]. Once activated, induction to transcription factor such as c-Jun, c-fos and c-myc at nucleus, is induction of protein synthesis and increased to cell size and cell number, and eventually induced hypertrophy and hypertension.

We have investigated the expression level of signaling molecules, and cardiac hypertrophy related molecules such as PKB/Akt and Cn in cardiac tissues of SHR and WKY following four different developmental stages (10 d, 4 wk, 8 wk and 16-wk-old after born). Subsequently, the critical role of Cn in developmental stage of hypertension by cardiac vascular hypertrophy was investigated in the hypertrophic signaling, such as mentioned above, which are distributed in whole cell compartments to transmit the downstream signaling in hereditary cardiac hypertrophy of SHR. In addition, to make sure of functional differences between normotensive WKY and hypertrophied SHR heart, M-mode and Doppler echocardiography were performed for noninvasive assessment of left ventricular (LV) function and dimensions.

Materials and Methods

Animals

Spontaneoulsy hypertensive rat (SHR), Wistar Kyoto rat (WKY) strain, weighing 200-250 g, were housed in an air-conditioned, light- and temperature controlled environment, and maintained on standard laboratory rat chow in cages. Throughout this study, rats were fed and watered *ad libitum*. All the animals had been fasted for 12 hr prior to sacrifice for experiment or surgery. In details, male SHR at 4-wk-old age and their age-matched control WKY rat, were purchased from Charles River Company (Japan). Male SHR weighing about 250 g (at 12 to 14 weeks of age) were used for experiments. They were starved overnight and anesthetized by intraperitoneal administration of 100 ug of sodium pentobarbital per g of body weight at 10 to 15 min before the experiments. At 8-wk-old-age, systolic blood pressure of SHR and their control WKY rats were measured using tail cuff Plethysmography as described previously [19]. The 8-wk-old-age SHR with systolic blood pressure higher than 150 mmHg, and their control WKY rats with systolic blood pressure lower than 110 mmHg, and their control WKY rat systolic blood pressure lower than 110 mmHg, were used for the hypertension studies.

Blood pressure measurement

Blood pressures were determined using tail cuffs and a programmed electrosphygmomanometer following the recommendations of the manufacturer (PowerLab/400, AD Instrument, Austalia). Systolic blood pressure measurements were made in conscious SHR and WKY rats by a noninvasive tail cuff method as described previously [11]. Basically, we performed consecutive measurements each day for 2-3 days for each rat. Blood pressure measurements were continuously monitored over 30 min.

Subcellular membrane fractionation

Left ventricular tissues were placed in homogenized buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA; 0.25 M sucrose) containing 1 mM PMSF. The tissue was homogenized first using a Polytron for 5-sec bursts at a setting of 5 and then 10 up- and down-strokes of a motor-driven Teflon pestle in a glass homogenization tube. The whole homogenate (WL) was centrifuged at 16,000×g at 4°C for 15 min, yielding a pellet of plasma membrane (PM). The initial supernatant was centrifuged at 48,000×g for 20 min, yielding a pellet of high density microsomal fraction (HDM) containing Golgi membrane etc. The supernatant was then recentrifuged at 212,000×g for 90 min, yielding a second pellet of low density microsomal fraction (LDM) containing ER etc, and the remaining supernatant was concentrated by Speed Vac (Beckman CO.) and used as cytosolic fraction (CYT).

Immunoblot analysis

Cardiac muscle of SHR was homogenized in 5 m*l* of Buffer A (5 mM HEPES, ph 7.5), 250 mM sucrose, 0.1 mM PMSF, 1 mM benzamidine, 10 mM aprotinin) by 20 strokes in a Dounse Homogenizer. After centrifugation (100×g for 10 min) the supernatant was sonicated for 20 sec. Sonicates were cleared in a microcentrifuge for 8 min at 800×g. Subcellular fractionation of the supernatant was performed by centrifugation at 100,000×g for 1 hr in ultracentrifuge(Beckman CO.). The membrane fraction containing pellet was resuspended in buffer B (10 mM Tris, pH 7.5, 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM EDTA, pH 7.5, 1 mM DTT) and sonicated for 20 sec. Equal amounts of cell lysates, typically 10-20 ug, were fractionated by SDS-polyacrylamide gel electrophoresis on 12.5% gels and transferred to methanol soaked polyvinylidene difluoride (PVDF) membranes at 150 mA for 90 min in a Bio-Rad mini Transblot System. Then, all of steps were done according to traditional western blot procedure. Antibody-antigen complexes were detected using the enhanced chemiluminescence (ECL) reaction system (Amersham Pharmacia Biotech, UK). Cn, Akt/PKB, and ERK antibodies (Transduction Lab, USA) were all used as 1:2,500 dilution.

Echocardiography

M-mode and Doppler echocardiography were performed for noninvasive assessment of left ventricular (LV) function and dimensions using previously described method [10].

Statistical analysis

Results were expressed as mean±SEM. Differences between groups were compared by Student's unpaired *t* test. A value of $P<0.05$ was used as a criterion for statistical significance.

Results

Experimental animal models for hypertension studies

Cardiac hypertrophy is defined as an increase in heart size resulting from increase in cardiomyocyte cells volume. Although it is initially an adaptive response that temporarily augments or maintains cardiac output, sustained cardiac hypertrophy is leading cause of the development of heart failure and sudden death in humans. To investigate the process of hypertension in developmental stage of SHR and WKY rats, if SHR get into more than 12 weeks after born as hereditary hypertension rats, get into stable high blood pressure status (Fig. 1). Our experiment model compares with WKY and SHR and can confirm that get into

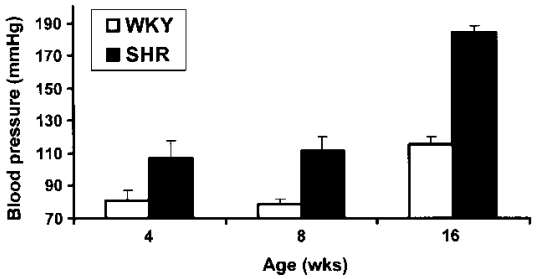

Fig. 1. Systolic blood pressure of WKY and SHR following developmental stages. WKY: Normotensive Wistar Kyoto Rat; SHR: Spontaneously Hypertensive Rat.

Table 1. Changes of body weight in WKY and SHR following developmental stages

	10d (n=10)	4 wk(n=6)	16 wk(n=2)
WKY	10.0 g±1.0	103.1 g±5.0	268.3 g±6.2
SHR	9.8 g±1.0	100.9 g±0.9	261.0 g±9.0

hypertension status as 16-wk-old (Fig. 1). By the way, according to WKY and SHR's growth process, weight could confirm that it is no significant differences between WKY and age-matched SHR (Table 1).

Expression of hypertrophy-related molecules, PKB/Akt and calcineurin in cardiac muscles of SHR and WKY rats following developmental stages

We have investigated the expression level of cardiac hypertrophy-related molecule such as PKB/Akt and Cn (Fig. 2). PKB/Akt expression showed similar pattern upon aging in cardiac muscle of WKY and SHR. But, the amount of total protein expression was almost twice higher in WKY rats (Fig. 2A). The protein expression of Cn in cardiac muscle of WKY rats showed no significant changes. However, in SHR, Cn expression of SHR was highest in 10 days and gradually decreased upon aging (Fig. 2B). These results showed that cardiac hypertrophy related molecule such as Akt and Cn were markedly decreased upon aging with developmental stage of cardiac hypertrophy in SHR compared to WKY rats, especially in the stable hypertensive 16-wk-old SHR. Total protein expression of Akt and Cn became much dramatically lower in SHR compared to WKY rats. Moreover, Cn was decreased at developmental stage of cardiac hypertrophy. Also, total protein expression became lower in SHR comparative WKY rats.

Expression and subcellular distribution of Cn, PKB/Akt, and ERK protein in cardiac tissues from 16-wks-old WKY rats and SHR

Sodium carbonate-based detergent-free cell lysates were prepared from cardiac tissues of SHR and WKY rats. To investigate the subcellular distribution of signaling molecules (Cn, PKB/Akt, and Erk) in WKY and SHR heart following developmental stages, subcellular membrane fractionations were performed as described in "materials and methods".

To examine colocalization of PKB/Akt, Erk, and Cn, subcellular membrane fractionation was performed. Equal amount of aliquots taken from each of the membrane fractions was subjected to immunoblotting with PKB/Akt, Erk, and Cn antibodies. As shown in Fig. 3, the results showed PKB/Akt expression was exclusively higher in WKY compared with SHR. This expression pattern was shown in Erk and Cn either. These results implicate that hypertrophy-related signaling molecules such as PKB/Akt, Erk, and Cn are importantly involved in development of hypertrophy with translocation to subcellular localization in SHR. Interestingly, expression

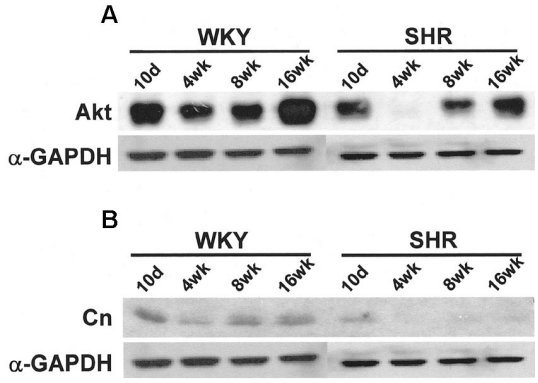

Fig. 2. Western blot analysis of PKB/Akt and calcineurin in cardiac tissue of WKY and SHR following developmental stages. Western blot of PKB/Akt and calcineurin expression from whole lysates of cardiac muscles was performed with a mouse monoclonal anti-Akt and calcineurin antibodies (A and B).

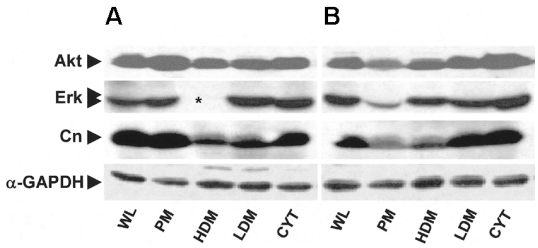

Fig. 3. Expression and subcellular distribution of hypertrophy-related signaling molecules (PKB/Akt, Erk, and calcineurin) in cardiac tissues from 16-wk-old WKY rats (A) and SHR (B). Whole lysates from cardiac tissues were subjected to subcellular fractination to yield each fraction enriched in plasma membrane (PM), high density microsomes (HDM), low density microsomes (LDM) and cytosol (CYT) fractions. Each fraction was analyzed by SDS-PAGE and immunoblotting with anti-Akt, Erk and calcineurin antibodies. *marker in Erk band of WKY rat tissue indicates strange missing band.

level of Erk was increased in whole cell lysate of cardiac tissues (Fig. 2) and left ventricle myocardium (Fig. 3) of SHR. However subcellular fractionation localization studies (Fig. 2) showed much lower translocation of Erk along with other signaling molecules (PKB/Akt, Cn) into plasma membrane in SHR. Furthermore, membrane fractionation indicated relatively lower translocation of Erk, PKB/Akt, and Cn in the PM (Fig. 3) from cytosol, or alteration in integrity of these molecules in cardiac tissues of SHR indicating a possible defect in their direct/indirect interaction (Erk - caveolin-3) in a sort of intracellular microdomain doing as shuttle molecule.

Echocardiography

Cardiac hypertrophy is defined as an increase in heart size resulting from an increase in cardiomyocyte cell volume. Although it is initially an adaptive response that temporarily augments or maintains cardiac output, sustained cardiac hypertrophy is a leading cause of the development of heart failure and sudden death in humans. Echocardiography provides a more accurate and sensitive means of detecting left ventricular hypertrophy than electrocardiography. To investigate the functional differences of hypertension in SHR with WKY rat heart, M-mode and Doppler echocardiography were performed for non-invasive assessment of left ventricular (LV) function and dimensions as described in “materials and methods”. In 16-wks-old SHR, 2-dimensional echocardiography showed cardiac enlargement (A-a and B-a) and hypertrophy of cardiac wall (A-b and B-b). Interestingly, the cause of cardiac enlargement and hypertrophy of cardiac wall in SHR compared to normotensive WKY rats was fibrosis of connective tissue of cardiac muscle (data not shown). This diagnostic technique therefore permits the identification of more subtle cardiac involvement, which nevertheless has prognostic importance.

Discussion

Primary hypertension is major risk factors for; i) persistent diastolic pressure 115 mmHg, ii) diabetes mellitus, iii) hypercholesterolemia, iV) obesity, V) cardiac damage; ischemia, myocardial infarction, congestive heart failure, Vi) kidney failure, Vii) nervous system disorder. The nature of the hypertrophic response of the heart is a compensatory response increased after load or disturbed function of the heart. This diagnostic technique therefore permits the identification of prognostic importance. Thus, the roles for positive or negative regulatory function of signaling molecules in the hypertensive heart will give us clue for the pathophysiology of hypertension suggesting necessary roles for a diverse of intracellular signaling pathway, which might be highlights the multi-functional nature of intracellular signal transduction pathway in cardiac hypertrophy. Hypertension is a major contributor to both macro-and microvascular disease, such as coronary artery disease, obesity and alcohol abuse [21]. The activation of membrane receptor is leads to activation of Ras-Raf-MEK-ERK pathway in transmitting these stimuli to the nucleus. Heterotrimeric GTP-binding proteins (G-protein) transduce stimulatory or inhibitory signals from hypertropic agonist (Endothelin-1, Angiotensin II) stimulation [5]. It is modulated the activity of downstream signaling effector, typically adenylyl cyclase (AC) or phospholipase C (PLC) [1]. In stably hypertensive 16-wks-old SHR (Table 1), two-dimensional echocardiography showed cardiac enlargement and hypertrophy of left ventricle (Fig. 4). The expression of PKB/Akt and Cn were down-regulated in cardiac tissues of hypertensive SHR compared to normotensive WKY rats (Fig. 2A, B). In addition, subcellular localization of hypertrophy-related signaling molecules (PKB/Akt, Erk, Cn) was potentially decreased in plasma membrane (PM) fraction of cardiac tissues from 16-wks-old SHR compared to WKY rats (Fig. 3). This observation suggests that retardation of the translocation of these molecules in the plasma membrane may be necessary to propagate the development of cardiac hypertrophy.

To understand the molecular determinants of the hypertrophic response, recent investigation has focused on identifying and characterizing intracellular signal transduction pathway in the heart [19]. Traditionally, discussion of intracellular signaling has been largely associated with the actions of protein kinases such as the mitogenic-activated protein kinases (MAPKs) or the actions of G proteins. Recently caveolin isoforms have been known to regulate these proteins [12, 18]. Thus, we examined interactions between the caveolin-3 and hypertrophy-related signaling molecules in the hypertensive SHR heart tissue (data not shown). It has been demonstrated that caveolin-3 binds to many growth factor receptors [9] and its downstream molecules such as PKB/Akt, Erk, leading to the activation of cellular growth. In summary, our data indicate that hypertrophy-

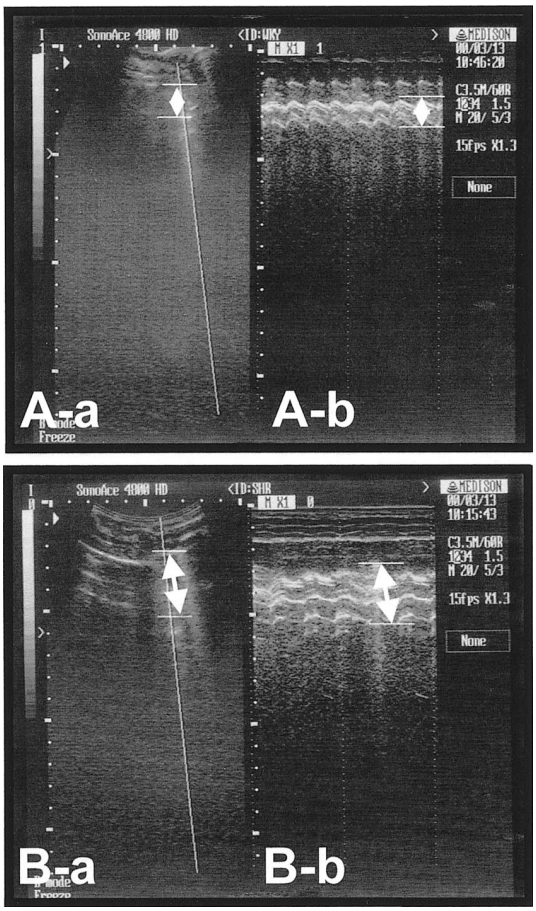

Fig. 4. Two-dimensional echocardiography of 16-wk-old WKY and SHR M-mode and Doppler echocardiography were performed for non-invasive assessment of left ventricle (LV) function and dimensions as described in "materials and methods". WKY rats (A) and SHR (B). A-a and B-a indicate heart size, and A-b and B-b indicate thickness of cardiac wall.

related molecules, PKB/Akt, Erk, and Cn are involved in cardiac hypertrophy, and suggest that PKB/Akt and Cn play as a negative regulator of cardiac hypertrophy in SHR compared to WKY rats.

On the contrary, our findings indicated that although systolic performance of the SHR ventricle is enhanced, this is due to the adequate hypertrophic response observed in this model that compensates for the increased wall stress these myocytes are subject to, which at this point, seems to have preserved inotropism. And also they never go to heart failure at each time point what we examined.

In conclusion, compared with WKY, the adult SHR has increased systolic performance with hypertrophic evidence in left ventricular wall as well as decreased the expression of signaling molecules such as PKB/Akt and Cn at 16-wk-old age point. Thus, The supernormal systolic function is due to a compensated hypertrophic response with preserved inotropism in SHR heart even if cellular expression and subcellular localization of signaling molecules are different between WKY and SHR hearts. However, we would like to emphasize that the data reported herein cannot necessarily be extrapolated to other hypertrophic and hypertension models.

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