Original Article

Noninvasive Evaluation of the Time Course of Change in Cardiac Function in Spontaneously Hypertensive Rats by Echocardiography

Manabu KOKUBO, Arata UEMURA, Tatsuaki MATSUBARA*,
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The spontaneously hypertensive rat (SHR) has been well established as a suitable model for studies of hypertension, but little is known about the processes of left ventricular (LV) hypertrophy and the changes in cardiac function in this model. The present study was designed to provide a noninvasive evaluation of the time-dependent alteration of cardiac function in male SHR at 4 to 24 weeks of age and age-matched Wistar-Kyoto rats (WKY). Echocardiographic studies were performed after blood pressure (BP) and heart rate (HR) were measured by a tail-cuff method. The body weight (BW) of SHR was lighter than that of WKY at all ages, and HR was consistently lower, with significantly elevated systolic BP from 4 weeks of age. In the echocardiographic study, LV mass at 4 weeks of age was similar between WKY and SHR, although the ratio of LV mass to BW was higher in SHR than WKY. The ejection fraction, fractional shortening (FS) and midwall FS did not differ between the two groups at 4 weeks, but after 8 weeks, these parameters were decreased in the SHR. The deceleration time was prolonged in SHR after 16 weeks and the *E*/*A* ratio was lowered at 12 weeks. We also analyzed the expression levels of calcineurin, which were found to be increased in both groups with age. These results suggest that calcineurin does not play a major role in the development of LV hypertrophy. Thus, in SHR, cardiac hypertrophy develops by 4 weeks of age, and systolic and diastolic dysfunction is evident at 2 to 3 months. (*Hypertens Res* 2005; 28: 601–609)

***Key Words*: ventricular hypertrophy, spontaneously hypertensive rat, echocardiography, left ventricular function, calcineurin**

Introduction

The development of left ventricular (LV) hypertrophy is an adaptive response to pressure overload (*1*). Clinically, sustained hypertrophy correlates with an increase in the mortality of cardiovascular disease (*2*), and often is an initial step in the process to congestive heart failure. Cardiac hypertrophy is also a risk factor for arrhythmia and sudden cardiac death (*3*). To develop therapeutic approaches to prevent LV hypertrophy, it is important to elucidate the precise mechanisms and/or time course of its development. One potential focal regulator of cardiomyocyte hypertrophy that responds to altered calcium handling is the calmodulin-activated serine/threonine protein phosphatase calcineurin. Numerous studies have established that the calcium-calcineurin signaling pathway plays a critical role in the development of cardiac hypertrophy (*4*, *5*).

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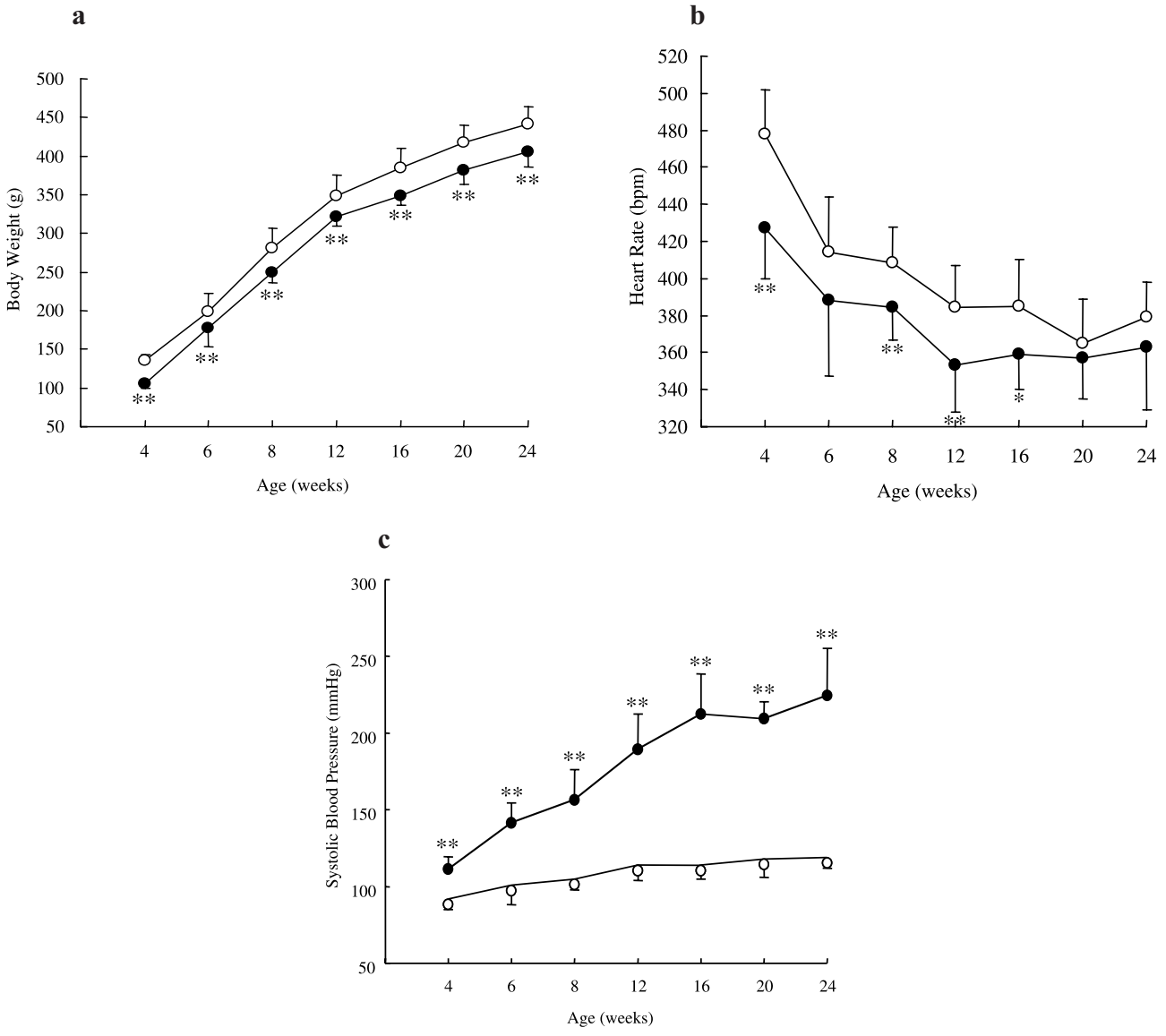

Fig. 1. *Changes in body weight (a), heart rate (b) and systolic blood pressure (c) for Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Data were obtained at 4, 6, 8, 12, 16, 20 and 24 weeks of age, and all values are the means±SD.* ○*, WKY ;* ●*, SHR.* *p < *0.05* vs. *WKY;* **p < *0.01* vs. *WKY.*

The hypertension in spontaneously hypertensive rats (SHR) is similar to human hypertension in numerous ways (*6*). One of these similarities, the occurrence of long-term, stable LV hypertrophy followed by a transition to heart failure, makes SHR a useful tool for studying the mechanisms of LV hypertrophy (*7*). While a number of studies on LV hypertrophy in SHR have already been performed, there have been only a limited number of studies on the time course of changes in LV hypertrophy and cardiac function in SHR.

Echocardiography is one of the most widely used noninvasive techniques to provide quantitative measurements of ventricular structure and function in humans and experimental animals. In rats, the heart is generally ~1 g in weight with a 2 mm LV wall thickness and a rapid heart rate of 300–400 bpm, and conventional transthoracic echocardiography transducers often do not provide enough resolution. However, high-frequency transducers (7.5–15 MHz) are now available to monitor morphometric and functional changes in experimental hypertrophy or myocardial infarction in small animals *in vivo* (*8*, *9*). The advantage of this approach is that it allows repeated noninvasive evaluation in a single live animal and serial determinations of cardiac structure and function to follow the disease progression and response to therapeutic interventions.

The main purpose of the present study was to perform a noninvasive evaluation of the time course of changes in car-

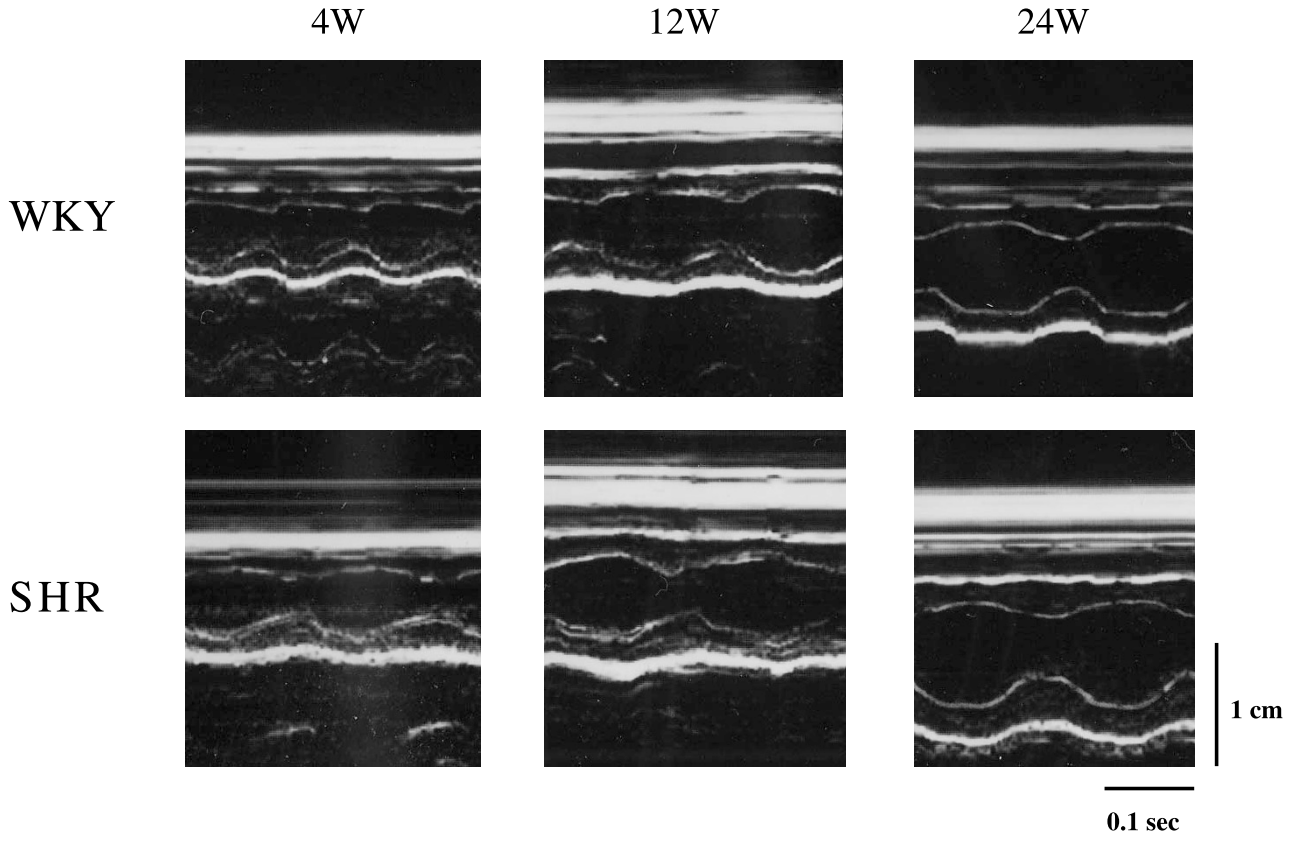

Fig. 2. *Examples of M-mode echocardiographic recordings for anesthetized Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) at 4, 12 and 24 weeks of age. Note the increased left ventricular (LV) thickness and LV end-diastolic and LV end-systolic dimension in the SHR group at 12 and 24 weeks of age. W, weeks of age.*

Table 1. Time Course of Changes in Data for Echocardiographic Parameters with M-Mode Measurements

Week	IVS (mm)	LVDd (mm)	LVDs (mm)	LVPWD (mm)	LV mass (g)	LV mass/BW (mg/g)	RWT	HR (bpm)	LV weight (g)
WKY									
4	1.02±0.11	6.00±0.50	3.03±0.36	1.09±0.14	0.86±0.04	6.36±0.30	0.35±0.04	379±27	0.69±0.05
6	1.07±0.08	6.88±0.31	3.87±0.46	1.13±0.16	0.93±0.06	4.73±0.46	0.33±0.05	354±20	0.80±0.09
8	1.20±0.20	7.15±0.38	4.23±0.34	1.29±0.16	1.06±0.09	3.79±0.51	0.36±0.05	340±19	0.92±0.03
12	1.22±0.16	7.50±0.33	4.14±0.38	1.33±0.24	1.10±0.09	3.14±0.23	0.35±0.06	313±36	1.01±0.09
16	1.33±0.13	7.63±0.56	4.22±0.50	1.36±0.18	1.15±0.12	3.01±0.34	0.38±0.09	308±40	1.08±0.07
20	1.36±0.11	7.83±0.36	4.50±0.46	1.40±0.18	1.21±0.09	2.91±0.31	0.37±0.08	307±24	1.12±0.09
24	1.39±0.07	7.87±0.21	4.37±0.24	1.44±0.09	1.21±0.02	2.72±0.19	0.36±0.03	301±19	1.19±0.07
SHR									
4	1.10±0.11	6.06±0.48	3.41±0.32*	1.10±0.20	0.89±0.06	8.38±0.88**	0.37±0.07	302±25**	0.72±0.02
6	1.35±0.17**	6.41±0.54*	3.63±0.46	1.55±0.29**	1.06±0.11*	6.03±0.64**	0.47±0.09**	302±26**	0.92±0.03**
8	1.69±0.16**	7.26±0.58	4.58±0.58	1.73±0.35**	1.30±0.16**	5.23±0.74**	0.48±0.10**	291±27**	1.14±0.07**
12	1.74±0.28**	7.59±0.30	4.80±0.30**	1.97±0.20**	1.44±0.10**	4.47±0.39**	0.51±0.05**	281±23**	1.29±0.06**
16	1.77±0.09**	7.92±0.58	4.89±0.60**	2.01±0.23**	1.55±0.16**	4.40±0.53**	0.52±0.08**	278±18**	1.42±0.03**
20	1.75±0.09**	8.46±0.59**	5.25±0.36**	1.94±0.13**	1.57±0.11**	4.08±0.37**	0.46±0.05**	277±16**	1.53±0.09**
24	1.93±0.13**	8.32±0.52**	5.25±0.62**	2.09±0.18**	1.67±0.12**	4.21±0.45**	0.52±0.08**	256±23**	1.63±0.12**

All data are mean±SEM. WKY, Wistar-Kyoto rat; SHR, spontaneously hypertensive rat; IVS, intraventricular septal wall; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; LVPWD, left ventricular posterior wall thickness; LV mass/BW, ratio of left ventricular mass to body weight; RWT, relative wall thickness; HR, heart rate during the performance of the echocardiographic study; LV weight, left ventricular weight. *$p<0.05$ *vs.* WKY; **$p<0.01$ *vs.* WKY.

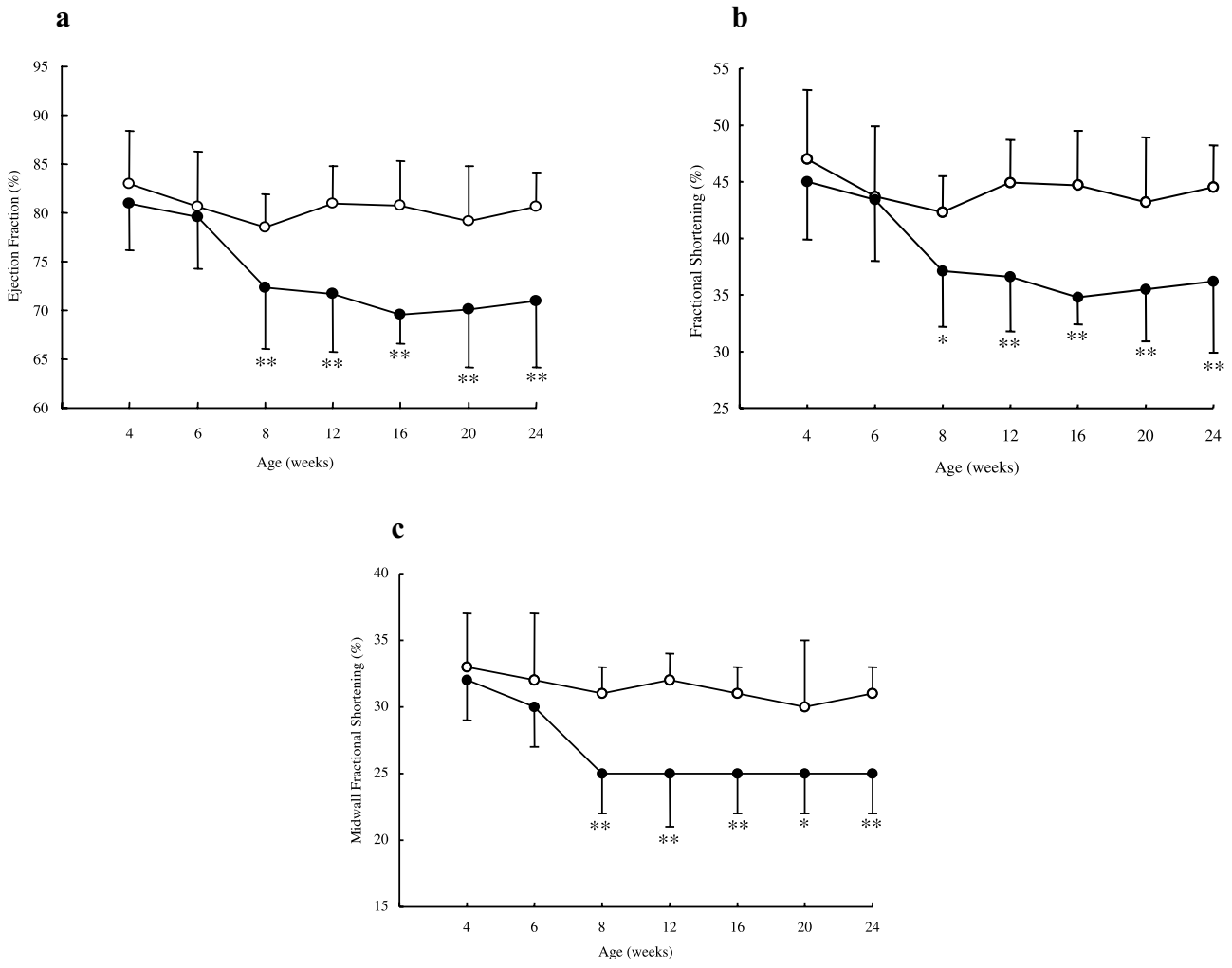

Fig. 3. *Changes in (a) ejection fraction (EF), (b) fractional shortening (FS) and (c) midwall FS for Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Data were obtained at 4, 6, 8, 12, 16, 20 and 24 weeks of age, and all values are the mean±SD. Note the decrease in EF, FS and midwall FS in the SHR group from 8 weeks of age.* ○*, WKY;* ●*, SHR.* *p < *0.05* vs. *WKY;* **p < *0.01* vs. *WKY.*

diac structure and function of SHR and age-matched Wistar Kyoto rats (WKY) *in vivo* by echocardiography. In addition, we evaluated changes in the expression levels of calcineurin.

Methods

Subjects

Forty-five male SHR and 45 age-matched WKY were used in this investigation. The starting age was 4 weeks. All of the animals were maintained at the Nagoya University Animal Experiment Center in a specific pathogen-free facility under conditions of controlled temperature (23±2°C) and humidity (55±5%) with a 12-h artificial light and dark cycle. The rats were given free access to standard laboratory chow and tap water. All procedures were in accordance with institutional guidelines for animal research.

Experimental Protocol

Twelve SHR and twelve WKY were weighed, and their systolic blood pressure (SBP) and heart rate (HR) were measured by a tail-cuff method (MK-2000, Muromachi, Tokyo, Japan) while conscious at 4, 6, 8, 12, 16, 20 and 24 weeks of age. After body weight (BW) and hemodynamic data were assessed, the echocardiographic studies were conducted. For measurement of LV weight and determination of calcineurin expression in cardiomyocytes by Western blotting, 4–6 SHR and 4–6 WKY were sacrificed at each of the above time points.

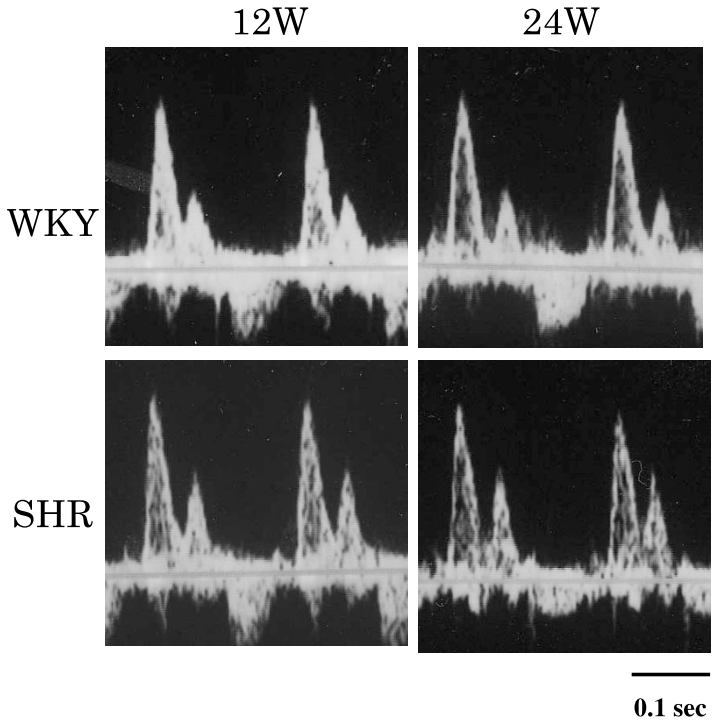

Fig. 4. *Transmitral inflow patterns for Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) at 12 and 24 weeks of age. Note the prolongation of deceleration time and increased A waves in the SHR group. W, weeks of age.*

Table 2. Transmitral Flow Measurements for the WKY and SHR Groups

Week	E-wave (cm/s)		A-wave (cm/s)		*E*/*A*		Decelertion time (ms)	
	WKY	SHR	WKY	SHR	WKY	SHR	WKY	SHR
4	101.2±11.0	99.3±9.1	—	—	—	—	41±5	41±4
6	100.7±9.2	97.3±7.3	—	—	—	—	43±7	44±4
8	103.2±8.5	99.6±13.2	—	—	—	—	44±4	44±5
12	100.2±9.7	99.6±15.0	43.9±4.4	52.1±8.5*	2.22±0.17	1.82±0.15**	42±7	46±8
16	97.5±7.6	95.2±8.9	42.9±3.8	53.2±6.7**	2.26±0.27	1.80±0.13**	42±6	47±6*
20	100.0±7.8	96.8±4.6	42.9±3.4	54.1±6.9**	2.27±0.18	1.81±0.23**	41±6	50±7**
24	97.9±8.3	97.1±7.7	43.0±3.3	52.6±9.4**	2.29±0.28	1.83±0.19**	41±8	52±4**

All data are mean±SD. WKY, Wistar-Kyoto rat; SHR, spontaneously hypertensive rat; *E*/*A*, ratio of peak early diastolic filling velocity to peak velocity at atrial contractions. *$p<0.05$ *vs.* WKY; **$p<0.01$ *vs.* WKY.

Echocardiographic Studies

Echocardiographic studies were performed between 4 and 8 PM with the animals in the left lateral decubitus position, after being anesthetized with ketamine hydrochloride 50 mg/kg and xylazine 10 mg/kg intraperitoneally. A Sonos 5500 echocardiographic system (Philips Medical Systems, Andover, USA) was used; the system was equipped with a 12 MHz transducer that was placed on the shaved left hemithorax.

Short axis views of the LV at or just below the tip of the mitral valve leaflets were used to obtain targeted M-mode recordings. Midwall fractional shortening (FS) was calculated as previously described (*10*).

The LV geometry of each rat at each age was classified into the following four geometric patterns according to LV mass/BW and relative wall thickness (RWT), as in an earlier report (*11*): concentric LV hypertrophy (increased LV mass/BW and increased RWT), eccentric LV hypertrophy (increased LV mass/BW and normal RWT), concentric remodeling (normal LV mass/BW) and normal geometry (normal LV mass/BW and normal RWT).

The transmitral flow velocity profile was determined by positioning a sample volume at the tip of the mitral valve on the para-apical long-axis view. The Doppler beam was set within 30° of the incident angle to the flow direction identi-

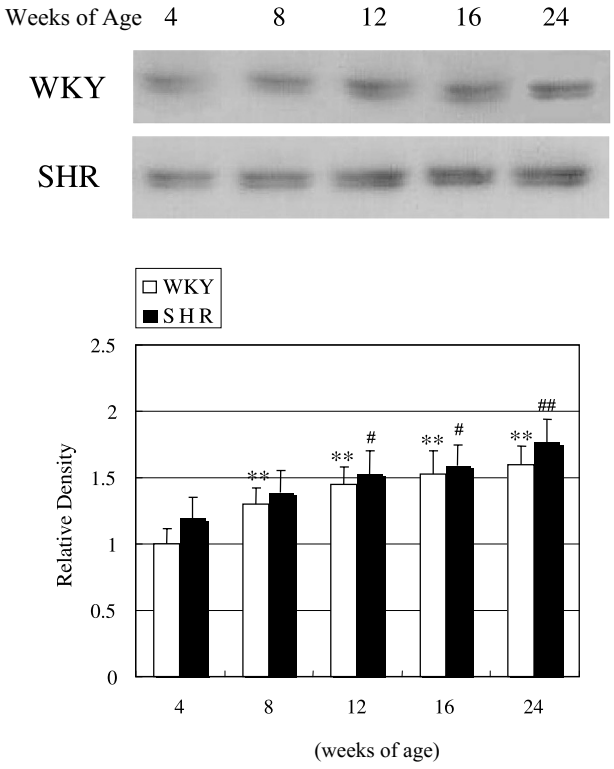

Fig. 5. *Western blot analysis of calcineurin in Wistar-Kyoto rat (WKY) and spontaneously hypertensive rat (SHR) hearts. Data are the means±SEM. Note the increase of the expression level of calcineurin with age, and the lack of statistically significant differences between age-matched WKY and SHR.* *p < *0.05* vs. *WKY at 4 weeks of age;* **p < *0.01* vs. *WKY at 4 weeks of age;* #p < *0.05* vs. *SHR at 4 weeks of age;* ##p < *0.01* vs. *SHR at 4 weeks of age.*

fied on color Doppler images. All data given are the means for 3 consecutive cardiac cycles.

Western Blot Analysis

Four to six SHR and four to six WKY were sacrificed at 4, 8, 12, 16 and 24 weeks of age by intraperitoneal injection of pentobarbital (100 mg/kg), and the heart ventricles were quickly separated, frozen, and stored at −70°C for the subsequent biochemical assays. The frozen hearts were weighed, minced and homogenized in 5 volume buffer (in mmol/l, Tris-HCl [pH 7.5] 30, DTT 1, EDTA 1, EGTA 1, leupeptin 0.02, and phenylmethanesulfonyl fluoride 0.2). Homogenates were centrifuged at 1,000×*g* for 15 min, and the supernatants were then centrifuged at 100,000×*g* for 1 h at 4°C to separate the cytosolic fraction (*12*).

Aliquots of protein from the cytosolic fractions were separated by SDS-PAGE, which was performed according to the method of Laemmli on 10% acrylamide gels (*13*), and transferred electrophoretically onto Immobilon P (Millipore) membranes. The membrane was blocked with 1% skim milk in phosphate-buffered saline (PBS, pH 7.3) and then incubated with anti-calcineurin monoclonal antibodies (Transduction Laboratories, Greenland, USA) diluted 500-fold. Unbound antibody was removed by washing in PBS containing 0.05% Tween 20. After incubation with sheep anti-mouse IgG conjugated to peroxidase at a 500-fold dilution as the secondary antibody, immunoreactive proteins were detected using as ECL Western blotting detection kit (Amersham Biosciences, Piscataway, USA).

Statistical Analysis

Data are expressed as the mean±SD. Statistical differences were determined by analysis of variance with StatView software. Unpaired *t*-tests were employed to compare data between age-matched WKY and SHR. A probability value of $p < 0.05$ was considered statistically significant.

Results

Two of the age-matched WKY died during the study, one at 12 and one at 16 weeks of age; in both cases, the animal died immediately following anesthesia and before echocardiography. The data from these two rats were excluded from the analysis.

Body Weight, Heart Rate and Blood Pressure

The BWs of SHR were lighter than those for WKY at all ages, and the HR was consistently lower in the SHR group. SHR demonstrated significantly elevated SBP compared to WKY from 4 weeks of age, and the SBP was persistently high (>200 mmHg) after 16 weeks (Fig. 1).

Echocardiographic Study

M-Mode Measurements

M-mode echocardiographic findings are shown in Fig. 2 and summarized in Table 1. At 4 weeks of age, the LV mass was similar in each group, but the LV mass to BW ratio was significantly greater in the SHR. After 8 weeks, the LV mass in SHR was significantly enlarged. To compare the accuracy of echocardiography, we also measured the LV weights of sacrificed rats. There was a tendency for the LV mass estimated by echocardiography to be heavier than the actual weights at all ages. The processes of LV hypertrophy development were similar, and the correlation coefficients for SHR and WKY were 0.95 and 0.98, respectively.

At 4 weeks of age, the LV geometry of SHR demonstrated an eccentric LV hypertrophy associated with an increased LV mass/BW and a normal RWT. After 6 weeks of age, the RWT of SHR progressively increased and concentric LV hypertrophy was demonstrated.

The data for ejection fraction (EF) and FS were not signifi-

cantly different between the groups at 4 weeks (EF: 80.9±4.8% for SHR *vs.* 83.0±5.4% for WKY; FS: 44.9±5.1% for SHR *vs.* 47.1±6.1% for WKY), but the values were decreased in the SHR group after 8 weeks (EF: 72.3±6.2% for SHR *vs.* 78.5±3.4% for WKY, $p<0.01$; FS: 37.1±4.9% for SHR *vs.* 42.3±3.2% for WKY, $p<0.01$). LV midwall FS was also calculated to avoid overestimation of the systolic function in the hypertrophied heart. There were no significant differences in the midwall FS between the two groups before 8 weeks of age (Fig. 3). These parameters in the SHR group were markedly decreased at 8 weeks and remained at the same level thereafter.

Doppler Measurements

Transmitral flow patterns for SHR and WKY at 12 and 24 weeks of age are shown in Fig. 4, and all data from Doppler measurements are summarized in Table 2. Because it was impossible to separate the early diastolic filling wave and late filling wave of transmitral flow, it was found necessary to exclude the data before 12 weeks of age.

The deceleration time was prolonged in the SHR group after 16 weeks. While there was no significant difference in the peak velocity between SHR and WKY, the late filling wave velocity in the SHR group was faster than that in the WKY group and the *E*/*A* ratio was lower.

Western Blot Analysis

To assess one possible factor inducing cardiac hypertrophy in SHR, we performed Western blotting against calcineurin. There was a tendency for calcineurin expression to increase with age in both groups (Fig. 5). However, there were no significant differences in the protein levels between age-matched WKY and SHR.

Discussion

The major finding of this echocardiographic study was that SHR already demonstrated cardiac hypertrophy at 4 weeks of age, with a decrease in systolic function at 8 weeks of age and diastolic dysfunction developing at 3 months after birth.

An early increase of LV weight was also observed in SHR of the present study, and previous reports have demonstrated that sympathetic nerve stimulation might play an important role in early stage LV hypertrophy (*14*, *15*). Furthermore, Dang *et al*. (*16*) showed that LV tissue angiotensin-II concentrations were significantly higher in SHR than in WKY from 15 to 28 weeks of age, although plasma angiotensin-II levels were similar in both groups. Angiotensin-converting enzyme activities in the SHR hearts may be important targets in terms of the ability of angiotensin-converting enzyme inhibitors to inhibit cardiac hypertrophy (*17*). These data suggested that the renin-angiotensin system in SHR as well as sympathetic nerve stimulation might contribute to LV hypertrophy.

During the development of hypertension, alterations in LV geometry may occur as an adaptation to increasing pressure and volume load. In hypertensive patients, LV geometry can be classified into four patterns on the basis of LV mass index and RWT (*11*), and these patterns have been shown to be closely related to the LV function and to patients' prognosis (*18*, *19*). In the present study, eccentric LV hypertrophy was apparent at the first measurement time point in the SHR, and after 6 weeks, RWT increased progressively, a geometric pattern conforming to concentric hypertrophy. These early geometric changes might play some role in compensating and maintaining LV function.

Regarding earlier work on cardiac function in SHR, Peffer and Frolich (*20*) demonstrated an increased cardiac output in young (9- to 12-week-old) individuals of this model. They also reported that this increase disappeared with pharmacological inhibition of extrinsic autonomic input to the heart. Other investigators found increased contractility in myocytes isolated from the SHR heart (*21*, *22*). Hemodynamic measurements using a catheter pressure transducer *in vivo* have shown increased systolic performance of SHR hearts (*22*, *23*). However, there have also been reports that contractility was not changed in these animals (*24*, *25*). There are many factors affecting cardiac function *in vivo*, such as humoral agents, adrenergic stimulation, the interstitium, and the coronary circulation. Since systolic indices of echocardiographic study are dependent on preload and afterload (*26*), the present finding that SBP rose suddenly in SHR at 8 weeks of age suggests that increased afterload may depress EF, FS and midwall FS in the SHR heart. Pathological cardiac hypertrophy causes a breakdown of the balance of the energy metabolic system in the heart. It has been described that the energy production in cardiac hypertrophy in SHR showed a shift from the use of fatty acids toward the use of glucose (*27*). Iemitsu *et al*. showed that the mRNA expression of key enzymes in the glycolytic metabolic pathway was markedly increased in the SHR hearts, indicating a decline of cardiac function (*28*). Diastolic dysfunction is to be expected in the SHR because of the increased wall thickness and fibrosis (*29–31*). Okayama *et al*. (*22*) reported that relaxation was not altered in isolated myocytes of SHR at 10 weeks of age, but Nishimura *et al*. (*29*) analyzed LV diastolic function using τ and demonstrated prolongation at 28 and 50 weeks of age compared with age-matched WKY. In the same way, Cingolani *et al*. (*23*) showed an increase in τ in adult SHR (10 to 11 months old) using a catheter pressure transducer. It is unclear when diastolic dysfunction develops in the SHR heart, but at 10 weeks of age the length and diameter of cardiomyocytes are larger than normal (*22*) and there is increased collagen content in the LV wall (*32*). These data are in line with our finding that diastolic dysfunction of SHR was present early in life. To clarify the mechanisms of cardiac dysfunction, further investigation in SHR is required.

Evaluation of the transmitral flow pattern of small animals is technically difficult, because their HRs are 300 to 400 bpm and the E and A waves fuse (*33*). In the present study, there-

fore, we anesthetized the animals with ketamine hydrochloride and xylazine, which have the effect of decreasing HR to about 70% to 80% of the level in conscious animals. Our study demonstrated an increased A-wave velocity and prolonged deceleration time at 3 months after birth, pointing to a delayed relaxation pattern. These changes are seen very commonly in patients with a wide variety of cardiovascular disorders. However, there was no SHR with an *E*/*A* below 1.0, which is frequently seen in patients with hypertension and is a criterion for diastolic dysfunction in humans. The normal value of *E*/*A* is almost 2.0 in rats (*34*) .

Calcineurin is the one of the major proteins that plays an important role in the development of cardiac hypertrophy (*4*, *5*, *35*). Zou *et al*. (*36*) reported that the calcineurin activities in the hearts of SHR progressively increased with age. Some investigators have tried to prevent cardiac hypertrophy using calcineurin inhibitors, such as cyclosporin A and FK506, but were not successful (*37*, *38*). In the present study, calcineurin expression increased with aging, but there were no differences in the level of this protein between age-matched rats. Further investigation of this pivotal regulator for the development of cardiac hypertrophy in SHR will be needed.

A number of limitations of this study bear mentioning. It is well known that most anesthetics cause cardiovascular and respiratory system depression. Although ketamine has been reported to have less of a cardiodepressor effect, it is usually applied in combination with xylasine or diazepam to improve muscle relaxation and prolong analgesia (*39*, *40*). Such a combination has been shown to produce hypothermia and to have negative inotropic effects on the heart, and may have influenced our results.

In summary, the suitability of echocardiography for noninvasive evaluation of LV characteristics and systolic function in rats has been well established. Recent advances in echocardiography and the tail cuff method have allowed investigators to obtain accurate hemodynamic data, and in combination with Doppler transmitral flow patterns, these methods have provided essential data on diastolic function. Using this approarch we could have demonstrate cardiac hypertrophy development by 4 weeks of age in the SHR, systolic and diastolic dysfunction being evident at 2 to 3 months of age.

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