ALTERATIONS IN SARCOPLASMIC RETICULUM AND ANGIOTENSIN II RECEPTOR TYPE 1 GENE EXPRESSION IN SPONTANEOUSLY HYPERTENSIVE RAT HEARTS

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Left ventricular hypertrophy (LVH) is an adaptive change in response to hypertensive pressure overload. Some evidence indicates that the decrease in sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase mRNA expression, which may contribute to a diastolic dysfunction of the heart, occurs in the experimental pressure overload model. Also, recent studies have demonstrated that angiotensin II (Ang II) and angiotensin II receptor type 1 (AT1) play important roles in LVH. The purpose of this study was to investigate the function of the SR and the role of AT1 in genetic hypertension in spontaneously hypertensive rats (SHR) at ages 10 and 18 weeks. In SHR, cardiac hypertrophy has already developed at 10 weeks of age. SR Ca\(^{2+}\)-ATPase activity and mRNA expression were significantly lower in SHR than in Wistar-Kyoto rats (WKY). Plasma renin activity in SHR was unchanged compared with WKY, whereas the Ang II concentration in SHR was significantly higher than that in WKY. AT1 mRNA expression in SHR was similar to that in WKY. These results suggest that in the early stage of hypertension in SHR Ang II may stimulate hypertrophy in the cardiomyocytes through the AT1, which is not downregulated by a high concentration of Ang II. (Jpn Circ J 1999; 63: 367-372)

Key Words: Angiotensin II; Angiotensin II receptor type 1; Left ventricular hypertrophy; Sarcoplasmic reticulum Ca\(^{2+}\)-ATPase; Spontaneously hypertensive rat

The spontaneously hypertensive rat (SHR) is the most popular laboratory model of human essential hypertension.\(^1\)-\(^3\) A chronic pressure overload of hypertension causes left ventricular hypertrophy (LVH), and this is followed by a relatively long period of stable, compensated hypertrophy.\(^4\) LVH is characterized by an increase in myocyte size, the interstitial fibrosis that is responsible for the abnormal myocardial stiffness,\(^5\) and alterations in cardiac gene expression, including sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase (SERCA2a) that is a potent regulator of intracellular calcium handling.\(^6\) Hypertension and the renin-angiotensin system, particularly angiotensin II (Ang II), play an important role in LVH.\(^7\) Although the plasma renin activity of SHR did not increase, an angiotensin-converting enzyme (ACE) inhibitor decreased blood pressure and suppressed LVH.\(^8\)\(^,\)\(^9\) Ang II exerts its effects by means of membrane-bound angiotensin receptors.\(^10\) Ang II receptors are classified according to their pharmacological characteristics, and the 2 major subtypes are angiotensin II type 1 and type 2 (AT1 and AT2, respectively) based on their inhibition by Ang II receptor antagonists losartan for AT1 and PD123319 for AT2.\(^11\) Furthermore, the AT1 group consists of 2 subtypes, AT1a and AT1b, that share a 71% homology on the gene level and a 95% homology on the protein level.\(^12\) In the adult rat heart, it is thought that Ang II plays a significant role in the regulation of LVH by means of AT1.\(^13\) To study the pathophysiology of LVH, in which a diastolic function of the heart is impaired and ventricular hypertrophy progresses, we investigated changes in AT1 mRNA in parallel with changes in SR Ca\(^{2+}\)-ATPase activity and expression of SERCA2a mRNA in the early stage of hypertension in SHR.

MATERIALS AND METHODS

All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985). Male SHR and Wistar-Kyoto rats (WKY) were purchased from Charles River, Japan. Ten male animals of each strain were studied at 10 and 18 weeks of age, when cardiac hypertrophy had already developed. The rats were housed similarly under standardized conditions and were fed regular rat chow and tap water ad libitum. Systolic blood pressure and heart rate of the rats were measured by the tail-cuff method.

Each rat was anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg), and the body weight was recorded. Blood was taken from the abdominal aorta for measurement of the renin activity and for determining the concentration of Ang II in the plasma. The thorax was rapidly opened, and the whole heart was immediately excised and rinsed with cold saline. The left ventricle was separated from the right ventricle and the atria and was weighed.

PREPARATION OF SR

Microsomes enriched in SR were prepared from individual rat hearts according to the method of Harigaya and Schwartz\(^14\) that was modified by Konno et al.\(^15\) Briefly, the left ventricular tissue was minced and homogenized with a

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Polytron homogenizer (Kinematica, Luzern, Switzerland) in 10ml of isolation medium containing 10mmol/L NaHCO3 and 5 mmol/L Na3. The homogenate was centrifuged for 10min at 9,000 × g (Himac CR21, Hitachi, Tokyo, Japan) and the resulting supernatant was collected and centrifuged for 10min at 12,000 × g. This supernatant was ultracentrifuged for 30min at 120,000 × g (55P-72, Hitachi), and the resulting pellet was suspended in 20mmol/L N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) buffer (pH7.0) containing 600mmol/L KCl and was then ultracentrifuged for 30min at 120,000 × g. The final pellet was resuspended in 20mmol/L TES (pH7.0) containing 50mmol/L KCl and the protein concentration was adjusted to 1 mg/ml. The protein concentration was determined according to the method of Lowry using bovine serum albumin as a standard.

**Measurement of Ca2+-ATPase activity of the SR**

Ca2+-ATPase activity was determined according to a modification of the method that uses an end-point assay of liberation of inorganic phosphate, as described previously by O’Brien et al.16 SR preparations (100 μg/ml) were homogenized in 10ml of isolation medium containing 10mmol/L NaHCO3 and 5 mmol/L Na3. The homogenate was centrifuged for 10min at 9,000 × g (Himac CR21, Hitachi, Tokyo, Japan) and the resulting supernatant was collected and centrifuged for 10min at 12,000 × g. This supernatant was ultracentrifuged for 30min at 120,000 × g (55P-72, Hitachi), and the resulting pellet was suspended in 20mmol/L N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) buffer (pH7.0) containing 600mmol/L KCl and was then ultracentrifuged for 30min at 120,000 × g. The final pellet was resuspended in 20mmol/L TES (pH7.0) containing 50mmol/L KCl and the protein concentration was adjusted to 1 mg/ml. The protein concentration was determined according to the method of Lowry using bovine serum albumin as a standard.

**Polymerase Chain Reaction (PCR) Analysis of AT1**

Total RNA (1μg) was reverse transcribed into single-stranded cDNA using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim). The following primers were used: AT1 upstream primer, 5'-TGCGAGTCTATACCGCTATGG-3'; AT1 downstream primer, 5'-AATATTGGTGCGGGGACCCAG-3'; GAPDH upstream primer, 5'-GCCATCAACGACCCCTTCATTG-3'; GAPDH downstream primer, 5'-TGCAGTGAGTCCGGCCGTC-3'. Amplification of AT1 was carried out for 40 cycles of 94°C for 15s, 65°C for 15s, and 72°C for 45s. Amplification of GAPDH was carried out for 30 cycles of 94°C for 15s, 62°C for 15s, and 72°C for 45s. Ten microliters of the PCR reaction was electrophoresed on a 2% agarose gel and stained with ethidium bromide. Identification of the AT1 and GAPDH transcripts was confirmed by the presence of 525-bp and 598-bp RT-PCR products. Quantities of AT1 and GAPDH DNA were determined by UV transillumination of the gel. AT1 mRNA expression was normalized by the GAPDH mRNA value for statistical analysis.

**Histological Analysis**

Sections of left ventricular free wall and septum were fixed in 10% formalin neutral buffer solution (pH7.4) and embedded in paraffin for microscopy and histological analysis. Myocardial transverse diameters were measured at the level of a nucleus with hematoxylin and eosin staining. Areas occupied by interstitial fibrosis were estimated by the point-counting method using Mallory-Azan staining.

**Statistical Analysis**

All data are expressed as means ± SD. The unpaired Student’s t-test was used to compare individual experimental groups with the control group. A value of p<0.05 was considered to be statistically significant.

**Results**

**Blood Pressure and Hypertrophy**

The systolic blood pressure of SHR increased with age and was significantly higher than that of age-matched WKY at 10 weeks (p<0.05) and at 18 weeks (p<0.01). The heart rate was similar in SHR and WKY when the rats were 10 weeks old and when they were 18 weeks old. The left ventricular weight, normalized to body weight, in SHR was significantly greater than that in WKY at 10 weeks old (p<0.01) and at 18 weeks old (p<0.05) (Table 1).

**Renin Activity and Angiotensin II Concentration**

Plasma renin activities were not significantly different between the SHR and the WKY at 10 weeks old or at 18 weeks old. In contrast, Ang II concentrations were significantly higher in SHR than in matching WKY at 10 weeks old and when they were 18 weeks old. The left ventricular weight, normalized to body weight, in SHR was significantly greater than that in WKY at 10 weeks old (p<0.01) and at 18 weeks old (p<0.05) (Fig 1).
Alterations in SR and AT1 Gene Expression in SHR

Fig 1. (A) Plasma renin activity (PRA) in SHR and WKY and (B) plasma angiotensin II concentration (Ang II) in SHR and WKY. Black bars represent SHR and gray bars represent WKY. Data are expressed as means ± SD (n=10). *p<0.05, **p<0.01.

Fig 2. Ca\textsuperscript{2+}-ATPase activity of sarcoplasmic reticulum from left ventricles of SHR and WKY. Black bars represent SHR and gray bars represent WKY. Data are expressed as means ± SD (n=10). *p<0.05.

Fig 3. (A) Sarcoendoplasmic reticulum Ca\textsuperscript{2+}-ATPase 2a (SERCA2a) mRNA expression by Northern blot analyses from left ventricles of SHR and WKY. β-Actin mRNA expressions are used as internal controls. (B) The value normalized by β-actin is expressed as the SERCA2a/β-actin mRNA ratio. Black bars represent SHR and gray bars represent WKY. Data are expressed as means ± SD (n=10). *p<0.05.
Ca\(^{2+}\)-ATPase Activity and SERCA2a mRNA Expression

Ca\(^{2+}\)-ATPase activities were significantly lower in SHR than in age-matched WKY at 10 weeks old (4.98±0.51 vs 5.78±0.74 \(\mu\)mol Pi · mg protein\(^{-1}\) · h\(^{-1}\); p<0.05) and at 18 weeks old (4.71±0.27 vs 5.64±0.14 \(\mu\)mol Pi · mg protein\(^{-1}\) · h\(^{-1}\); p<0.05) (Fig 2). The ratio of SERCA2a to \(\kappa\)-actin mRNA expression was also significantly lower in SHR than in WKY at the ages of 10 and 18 weeks (p<0.05) (Fig 3).

AT\(_1\) mRNA Expression

AT\(_1\) mRNA expression of the SHR was not different between the strains at 10 and at 18 weeks of age. The ratio of AT\(_1\) to GAPDH was also unchanged between the SHR and the WKY (Fig 4).

Histology

Myocardial transverse diameters significantly increased in SHR at the ages of 10 weeks (p<0.01) and of 18 weeks (p<0.05) compared with age-matched WKY. Areas occupied by interstitial fibrosis significantly increased in SHR when they were 10 weeks old (p<0.01) and when they were 18 weeks old (p<0.05) (Fig 5).

Discussion

In the compensated cardiac hypertrophy of SHR, we found that the Ang II concentration significantly increased but AT\(_1\) mRNA expression was unchanged, and also that SR Ca\(^{2+}\)-ATPase activity and mRNA expression significantly decreased. We studied the rats when they were 10

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Fig 4. (A) Angiotensin II receptor type 1 (AT\(_1\)) mRNA expression by RT-PCR from the left ventricles of SHR and WKY. GAPDH mRNA expressions are used as internal controls. The presence of AT\(_1\) and GAPDH transcripts is confirmed by the 525-bp and 598-bp products between 692 bp and 501 bp of the molecular weight marker. (B) The normalized value is expressed as the AT\(_1\)/GAPDH mRNA ratio. Black bars represent SHR and gray bars represent WKY. Data are expressed as means±SD (n=10).

Fig 5. (A) Myocardial transverse diameter in SHR and WKY and (B) areas occupied by interstitial fibrosis in SHR and WKY. Black bars represent SHR and gray bars represent WKY. Data are expressed as means±SD (n=10). *p<0.05, **p<0.01.
weeks old and expected findings to show that the beginning of hypertension and cardiac hypertrophy was different from that of the compensated hypertrophy when the rats were 18 weeks old. However, our main determinations both at 10 weeks and at 18 weeks of age were the same.

In SHR, persistent hypertension begins when the rats are about 8 weeks of age. At 10 weeks of age, cardiac hypertrophy has already developed and continues as stable and compensated hypertrophy for a relatively long period. At about 72 weeks, left ventricular pump failure and muscle dysfunction appear, and at 96 weeks in most animals LVH progresses to overt heart failure. Marked myocardial fibrosis, increased passive stiffness, and impaired contractile function are important in the transition from compensated hypertrophy to heart failure in SHR. In the present study, at 10 weeks of age, systolic blood pressure increased, and the left ventricular weight to body weight ratio and myocardial transverse diameters increased. From these data, we reconfirmed LVH in the early stage of hypertension in SHR. Areas occupied by interstitial fibrosis also increased at 10 weeks old. Myocardial fibrosis began at an early stage of hypertension.

Previous studies provide evidence that cardiac contractile and relaxation functions may be disturbed at the level of the SR in cardiac hypertrophy induced by pressure overload. Such alterations in SR function are considered primarily to be the result of changes in the gene expression of calcium-handling proteins. In particular, a decrease in SR Ca\(^{2+}\)-ATPase mRNA expression may contribute to a diastolic dysfunction by causing intracellular Ca\(^{2+}\) overload. Several investigators have reported that SR Ca\(^{2+}\)-ATPase mRNA expression and the level of SR Ca\(^{2+}\)-ATPase protein are diminished in pressure-overloaded hypertrophy. Other reports show that these parameters were enhanced or unchanged with hypertrophy. In addition, little information is available with regard to whether SR Ca\(^{2+}\)-ATPase mRNA expression is altered in genetic hypertensive hypertension. Ohta et al. reported that SR Ca\(^{2+}\)-ATPase mRNA expression in SHR was higher than in WKY at 9 weeks old, but there was no difference between the strains at 5, 11, 17 and 27 weeks old. Boluyt et al. reported that Ca\(^{2+}\)-ATPase mRNA expression in SHR was not significantly different from that in WKY in the left ventricles of hypertensive and failing hearts when the rats were 18–24 months old. Because reports on SR Ca\(^{2+}\)-ATPase mRNA expression in cardiac hypertrophy of SHR were variable, we re-examined SR Ca\(^{2+}\)-ATPase mRNA expression in SHR. In the present study, SR Ca\(^{2+}\)-ATPase activity and mRNA expression were significantly lower in SHR than in WKY.

LVH is considered to be an adaptive process in response to increased workload. Recent studies have demonstrated that Ang II is involved in the development of cardiac hypertrophy, in particular in LVH. This evidence is based primarily on the observation that ACE inhibitors induce regression of LVH both in experimental animal models and in hypertensive patients. We also reported that plasma renin activity of SHR did not increase, nevertheless an ACE inhibitor was able to prevent the development of LVH in SHR. Furthermore, recent studies suggest that tissue or vascular Ang II plays a more important role than circulating Ang II, which is produced by means of renin, than previously thought in the development of LVH. The present study, which showed that the plasma renin activity of SHR did not increase but Ang II concentration markedly increased, partly supports this hypothesis. That is, the high Ang II concentration in SHR was provided not only by means of renin but was also much more locally produced.

The mRNA level of AT\(_1\) was downregulated by Ang II in cultured rat glomerular mesangial cells. This finding suggests that Ang II itself is one of the potent regulators of AT\(_1\). Suzuki et al. reported that AT\(_1\) mRNA expression in SHR was unchanged compared with WKY at 17 weeks of age, but from 20 weeks AT\(_1\) mRNA expression was significantly increased. In the present study, although the Ang II concentration of the SHR markedly increased, the AT\(_1\) mRNA expression in the SHR was unchanged compared with age-matched WKY at 10 and at 18 weeks of age. The reason for the Ang II high concentration not downregulating AT\(_1\) mRNA expression in SHR was not determined in this study, but it may be related to the differences between glomerular mesangial cells and cardiomyocytes, between in vitro and in vivo, or between the physiological state and the pathological hypertrophic status. Further studies on the regulation of AT\(_1\) in SHR will be required.

Acute myocardial infarction causes a reactive hypertrophy of viable myocytes, in which AT\(_1\) mRNA expression is significantly increased. Previously, we reported that AT\(_1\) mRNA expression was increased in the non-infarcted myocardium, which was considered to be partly responsible for the hypertrophy and fibrosis in these areas whereas in LVH in the early stage of hypertension in SHR AT\(_1\) mRNA expression was unchanged. The AT\(_1\) antagonist TCV-116 induced regression of cardiac hypertrophy and had cardioprotective effects on hypertrophied myocardium in SHR. This result suggests that Ang II acts as a growth factor on cardiac myocytes by means of AT\(_1\). Although AT\(_1\) mRNA expression was unchanged, LVH developed in SHR. It may be suggested that Ang II continues to stimulate hypertrophy in the cardiomyocytes because of a high Ang II concentration.

We reported that an ACE inhibitor was able to increase SR Ca\(^{2+}\)-ATPase activity during a 1 h ischemic myocardium in a canine coronary ligation model. We also reported that an ACE inhibitor given to SHR from 10 to 18 weeks of age was able to increase SR Ca\(^{2+}\)-ATPase activity in these rats at 18 weeks old. These results demonstrate a protective effect of ACE inhibitors on SR Ca\(^{2+}\)-ATPase activity in ischemic myocardium and SHR. This effect may be related to the actions of ACE inhibitors, ie, inhibiting the production of Ang II, increasing bradykinin and prostaglandin and preventing enzyme oxidation. In our more recent study, we reported that AT\(_1\) blockade inhibited the reduction of SR Ca\(^{2+}\)-ATPase activity in non-infarcted myocardium in rats. These results suggest that Ang II may decrease SR Ca\(^{2+}\)-ATPase activity in SHR by means of AT\(_1\). Therefore, we conclude that in the early stage of hypertension in SHR Ang II may stimulate hypertrophy in the cardiomyocytes through the AT\(_1\) that is not downregulated by a high Ang II concentration.

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References
