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Reduced Activity and mRNA Level of the Na^+/Ca^{2+} Exchanger in Pressure-Overload Hypertrophy in Rat Hearts

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ABSTRACT

Little is known about the relationship between the function and gene expression of Na^+/Ca^{2+}-exchanger (NCX) in cardiac hypertrophy. Few experiments using cardiac hypertrophy models in the same disease stage have examined the correlations between NCX functions and changes in the levels of gene expression. In this study, we used whole hearts with moderate-to-severe hypertrophy from Goldblatt renovascular hypertensive rats and investigated the dynamics and physiological activity of the NCX by observing intracellular Ca^{2+} handling in the perfused hearts loaded with fluorescent Ca^{2+} indicator, fura-2. The correlation with genetic changes in the hypertrophied heart at the same disease stage was then examined. The following three results were obtained : (1) decreases in the velocities of intracellular Ca^{2+} influx and efflux induced by transient depletion of extracellular Na^+ ; (2) a decrease in NCX activity assessed by transient depletion of extracellular Na^+ with ryanodine treatment ; and (3) a decrease in messenger RNA expression of NCX in hypertrophic hearts. Impaired Ca^{2+} handling might be partially related to genetic changes of the NCX in cardiac hypertrophy. (Jikeikai Med J 2006 ; 53 : 111-20)

Key words : Ca^{2+} handling, cardiac hypertrophy, Goldblatt rat, Na^+/Ca^{2+}-exchanger, messenger RNA

INTRODUCTION

Progressive cardiac hypertrophy causes diastolic dysfunction, which is often observed in heart failure. Impaired muscular relaxation is a characteristic of hypertrophic myocardium. In patients with cardiomyopathy, the myocardium contracts and relaxes more slowly than does normal myocardium[1]. Because calcium ions (Ca^{2+}) in the cytoplasm play a central role in the process of excitation-contraction coupling, which is an important element in contraction and relaxation, prolonged cytoplasmic Ca^{2+} attenuation during the Ca^{2+} transient period is believed to lead to muscular relaxation impairement[2,3]. Experiments using cardiac myocytes isolated from hypertrophic hearts have shown prolonged attenuation of the intracellular Ca^{2+} transient[4,5]. These experiments have shown that changes in Ca^{2+} dynamics related to the prolonged relaxation of the myocardium in the hypertrophic heart. Na^+/Ca^{2+}-exchanger (NCX) and sarcoplasmic reticulum (SR) are the main regulatory mechanisms of intracellular Ca^{2+}. In the hypertrophic heart, Ca^{2+} uptake by the SR is slowed and may be related to inadequate myocyte relaxation or intracellular Ca^{2+} overload.

Gene targeting analysis has been used recently to elucidate the mechanisms of functional changes in the ionic transportation system. For example, 2

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isoforms of SR Ca^{2+}-ATPase (SERCA2) have been analyzed genetically, and some researchers have reported lower levels of this messenger (m) RNA in pressure-overloaded hypertrophied hearts[6–10]. However, other studies have suggested that the expression of these genes either increases[11,12] or does not change[13,14]. Some studies have suggested that genetic changes are related to the degree of hypertrophy in the heart. Specifically, SERCA2a mRNA expression increases in the mildly hypertrophic heart and decreases in the severely hypertrophic heart[8].

Another mechanism of functional change is Ca^{2+} release channels (ryanodine receptors) in the SR. Some experiments in aortic stenosis model rats[15] or cardiomyopathy model hamsters[16] have shown decreased expression of mRNA in the hypertrophic heart. In human, the mRNA expression of ryanodine receptors is suppressed in ischemic cardiomyopathy, but does not change in idiopathic cardiomyopathy[17]. One report states that severe heart failure decreases the density of Ca^{2+} release channels, and, as a compensatory mechanism, Ca^{2+} release channels become more sensitive[18].

Little is known about the functions and gene expression of NCX in cardiac hypertrophy, although many studies have shown that the expression of NCX is up-regulated and compensates for decreased expression of SERCA2a in heart failure. Furthermore, few experiments using models of cardiac hypertrophy in the same stage of the disease have examined the correlations between the exchanger functions and changes in the levels of gene expression. Studies in a rat model of cardiac hypertrophy showed no significant correlation between exchanger mRNA levels and exchanger activity[9].

Therefore, we investigated the dynamics and physiological activity of the NCX by observing intracellular Ca^{2+} handling in the hearts of Goldblatt rats with renovascular hypertension. The isolated hearts were perfused and loaded with a Ca^{2+}-sensitive fluorescent indicator, and the correlation with genetic changes in cardiac hypertrophy at the same stage was examined.

Materials and Methods

1. *Animals*

Ten 4-week-old Sprague-Dawley rats weighing 120 to 130 g were divided into 2 groups of 5 rats each. One group, the cardiac hypertrophy group, comprised Goldblatt model rats with renovascular hypertension. The other group was the sham-operated control group. All rats in both groups were anesthetized by intraperitoneal injections of 30 mg/kg sodium pentobarbital. Incisions were made through the skin in the left dorsal area to expose the left renal artery. The left renal arteries of the rats in the cardiac hypertrophy group were constricted with 0.35-mm-diameter silver clips. The sham-operated control rats underwent the same procedure without constriction. After each operation, blood pressure in the caudal artery was periodically measured with the tail cuff method over time. Six weeks after the operation, the heart was removed for a series of experiments. Care of the animals was based on the Animal Handling Policies of The Jikei University School of Medicine.

2. *Measuring cardiac function*

The heart was removed under anesthesia with intraperitoneal injection of 30 mg/kg sodium pentobarbital. The heart was then retrogradely perfused through the aorta with a constant flow of 14 ml/min using a peristaltic pump. Tyrode solution buffered with N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) was used as the perfusate and was maintained at 37°C in an aerobic condition by bubbling with 100% O_2 gas. A catheter was inserted into the left ventricle via the left atrium. Left ventricular pressure was measured with a polygraph system (Nihon Kohden, Tokyo). The heart rate was monitored with an electrode attached to the surface of the heart by surface tension.

3. *Intracellular Ca^{2+} measurement*

The intracellular Ca^{2+} concentration was measured by loading the heart with fluorescent dye, fura-

2-acetoxymethyl ester (fura-2, Dojindo Laboratories, Kumamoto). The details of this procedure have been previously described[19,20]. First, fura-2 was dissolved in dimethyl sulfoxide, after which 25 ml of Tyrode solution was added to produce a loading solution with a final fura-2 concentration of 4 μM. The heart was initially perfused for 15 minutes with standard Tyrode solution and then was perfused with loading solution, which was recirculated for 30 minutes to load the myocardium with the fluorescent dye. After loading, the heart was perfused again with standard Tyrode solution for 20 minutes to wash away extracellular fluorescent dye. The fluorescence was measured with optic fibers, a common probe, and an ion analyzer (CAF110 and CA200DP, Japan Spectroscopic Co., Japan). Ultraviolet lights from a xenon lamp excited the left ventricular myocytes through the fiber when the probe was attached to the surface of the left ventricle. Another fiber was used to collect the fluorescence from the ventricle. Intracellular Ca^{2+} was measured by the ratio of the fura-2 fluorescence intensity of 500 nm excited at 340 nm and 380 nm of UV light. Figure 1 shows the original recordings of 500-nm fluorescence intensity excited at 340 nm, 380 nm, the ratio, and the left ventricular pressure. Fluorescence intensity was not affected by motion artifacts produced by the beating heart. All variables were monitored simultaneously.

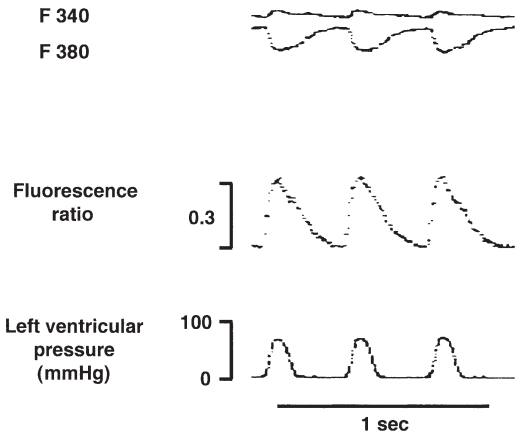

Fig. 1. Original traces in a spontaneously beating heart during standard perfusion. Upper, fura-2 fluorescence excited at 340 nm and 380 nm; middle, fluorescence ratio of 340 nm and 380 nm; lower, left ventricular pressure.

4. Ca^{2+} influx and efflux during Na^+-free perfusion

After being perfused with standard Tyrode solution, the heart was perfused for 20 seconds with a Na^+-free Tris solution (Na^+: 0 mM) in which NaCl was replaced by Tris aminomethane (Sigma-Aldrich, St. Louis, MO, USA). The solution was then changed again to standard Tyrode solution.

The depletion of extracellular Na^+ produces a

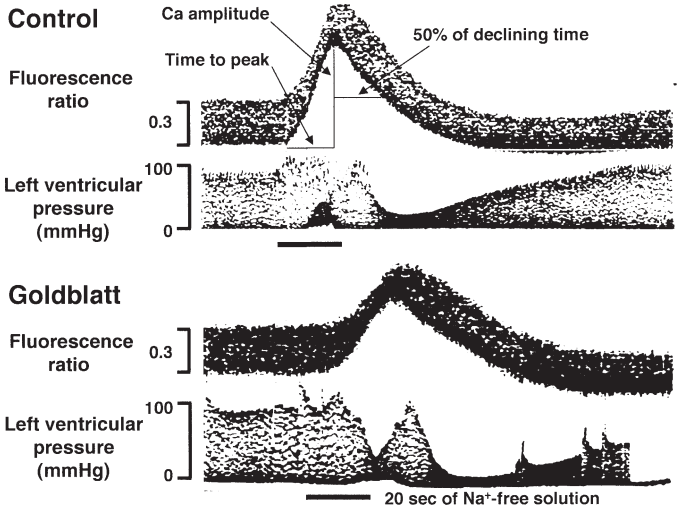

Fig. 2. Original traces of changes in intracellular Ca^{2+} and left ventricular pressure in sham-operated control (upper) and Goldblatt (lower) rats in constricted scale. When the heart was perfused with a Na^+-free solution for 20 seconds, Goldblatt rats showed a slower Ca^{2+} increase and Ca^{2+} decrease than did control rats.

gradient difference between the extracellular and intracellular Na^+ concentrations, causing Ca^{2+} influx due to the reverse driving force of NCX[21]. In addition to Ca^{2+} influx through NCX, Ca^{2+} is released from the SR through the Ca^{2+} release channel. As a result, cytoplasmic Ca^{2+} increases. When the heart is again perfused with standard Tyrode solution, the forward driving force of the NCX produces Ca^{2+} outward flux, the SR uptakes Ca^{2+}, and cytoplasmic Ca^{2+} decreases.

Figure 2 shows recordings of the fura-2 ratio during Na^+-free perfusion for 20 seconds. Measurements in both groups of rats included the time to peak diastolic Ca^{2+} elevation (time-to-peak), the difference between the baseline and peak diastolic Ca^{2+} (Ca^{2+} amplitude), the ratio of Ca^{2+} amplitude to the time-to-peak (rate of Ca^{2+} increase), 50% of the time required for diastolic Ca^{2+} to decline from the maximum level to the baseline level (50% of declining time), and the ratio of Ca^{2+} amplitude to 50% of declining time (rate of Ca^{2+} decrease).

We previously performed similar experiments with the L-type Ca^{2+} channel blocker, verapamil (1×10^{-6} M) and found no significant differences in the time-to-peak or the 50% of declining time between the standard perfusion and the treatment with verapamil (unpublished data).

When we added Ni^{2+} (1×10^{-3} M), an inhibitor of the reverse and forward modes of the NCX, the increase in Ca^{2+} was attenuated[20]. Therefore, this increase in Ca^{2+} may result from Ca^{2+} influx by driving the NCX in reverse mode, and Ca^{2+} influx may trigger Ca^{2+} release from the SR. Thus, a decline in Ca^{2+} may be caused by Ca^{2+} efflux through the forward driving force of the NCX and by uptake of Ca^{2+} into the SR. The direction of function of the NCX depends on the ionic gradient generated by the intracellular and extracellular Na^+ and Ca^{2+} concentrations.

5. Evaluation of Na^+/Ca^{2+} exchanger activity

To observe the functions of only the NCX, we used ryanodine, an inhibitor of the SR Ca^{2+} release channel. After the diastolic and systolic fluorescence ratios had been measured, the extracellular Na^+ was depleted to 0 mM for 20 seconds by replacing the standard perfusate with Tris solution. The Tris solution was subsequently replaced again with standard perfusate to confirm the return to the baseline condition. Then 1 μM ryanodine was added to the perfusate, and extracellular Na^+ was again depleted for 20 seconds. The solution was again replaced with standard perfusate. The time-to-peak, the rate of Ca^{2+} increase, the 50% of declining time, and the rate of Ca^{2+} decrease were measured as variables of influx and efflux of Ca^{2+} to indicate NCX activity.

Table 1. Oligonucleotide primers for NCX and GAPDH.

NCX	5′	GAACCCCGTCTGGTGGAGATGAGT
	3′	AAGAGAGTGACAGAGAAAGCTA
GAPDH	5′	TCCTGCACCACCAACTGCTTAGCC
	3′	TAGCCCAGGATGCCCTTTAGTGGG

6. Measurement of mRNA expression of the Na^+/Ca^{2+}-exchanger

From the hearts of age-matched rats, total RNA was extracted with the guanidium method[22] then subjected to reverse transcription (RT) using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Gene amplification with the polymerase chain reaction (PCR) was performed using Taq polymerase (Takara, Kyoto). The following were used as oligonucleotide primers: NCX[23]: 5′-GAACCCCGECTGGTGGAGATGAGT-3′, 5′-AAGAGAGTGACAGAGAAAGCTA-3′. glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[24]: 5′-TCCTGCACCACCAACTGCTTAGCC-3′; 5′-TAGCCCAGGATGCCCTTTAGTGGG-3′. PCR was performed under the following conditions for 25 cycles: denaturing at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. Under these conditions, after electrophoresis, the gene expression volume was quantified with imaging software (NIH Image, U.S. National Institutes of Health, Bethesda, MD, USA) (Table 1).

7. Statistics

Each value is expressed as a mean±SE. Student's *t*-test (unpaired) was used to determine signifi-

Table 2. Evaluation of hypertrophic hearts.

	Control	Goldblatt	Comparison
Body weight (kg)	0.47±0.26	0.38±0.04	not significant
Heart weight (g)	1.83±0.10	2.04±0.08	not significant
heart weight/body (g/kg)	3.97±0.17	5.72±0.44	$p<0.01$
Blood pressure (mmHg)	142±10	246±16	$p<0.01$
Left ventricular pressure (mmHg)	80.2±6.2	90.0±10.3	$p<0.05$
Heart rate (beats/min)	118±12	138±13	not significant

cance of differences between groups. Statistically significant differences were defined as having a p value <0.05.

Results

1. Evaluation of hypertrophy and hemodynamics

Although body weight was greater in control rats than in Goldblatt rats, there was no significant difference in heart weight between the groups. The heart weight to body weight ratio as an indicator of hypertrophy was significantly greater in Goldblatt rats than in control rats (Table 2). Goldblatt rats showed extremely high blood pressures. Moreover, under aerobic normal perfusion, the left ventricular pressure was greater in Goldblatt rats than in control rats. No significant difference was observed in heart rate between the groups.

2. Changes in intracellular Ca^{2+} concentration during Na^+-free perfusion

No significant differences were shown in the ratio of fura-2 fluorescence intensity in systole or diastole or in the amplitude of Ca^{2+} transient between the groups. Time-to-peak was significantly longer in Goldblatt rats (20.7±0.98 seconds) than in control rats (17.9±0.78 seconds ; $p<0.05$, Fig. 2 and 3).

The 50% of declining time was also significantly longer in Goldblatt rats (17.9±2.0 seconds) than in control rats (12.0±1.3 seconds ; $p<0.05$, Fig. 2, 3), suggesting either down-regulation of the forward mode of NCX or Ca^{2+} uptake into the SR in Goldblatt rats. The rate of Ca^{2+} increase was slightly but not significantly lower in Goldblatt rats (1.89±0.41) than in control rats (2.77±0.26 ; $p=0.09$). The rate of Ca^{2+} decrease was also significantly lower in Goldblatt rats (2.50±0.69) than in control rats (4.64±0.43 ; $p<0.05$, Fig. 4).

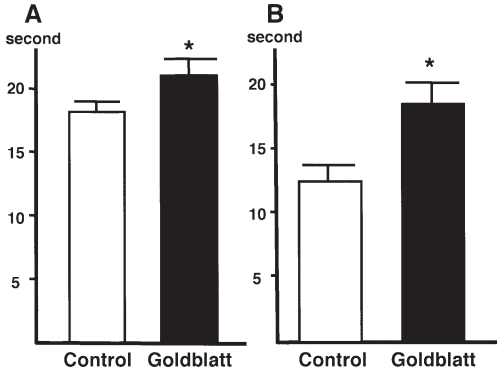

Fig. 3. Time-to-peak diastolic Ca^{2+} elevation (A), 50% of declining time from peak to baseline Ca^{2+} (B) during Na^+-free perfusion, $*p<0.05$ vs. control rats. $n=5$ for each group.

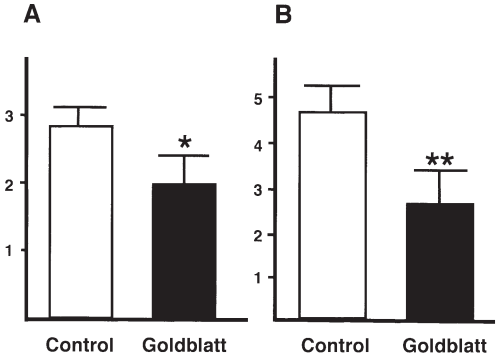

Fig. 4. Rate of Ca^{2+} amplitude to time-to-peak diastolic Ca^{2+} elevation (A), rate of Ca^{2+} amplitude to 50% of declining time from peak to baseline Ca^{2+} (B) during Na^+-free perfusion, $*p=0.09$ vs. control rats ; $**$ $p<0.05$ vs. control rats. $n=5$ for each group.

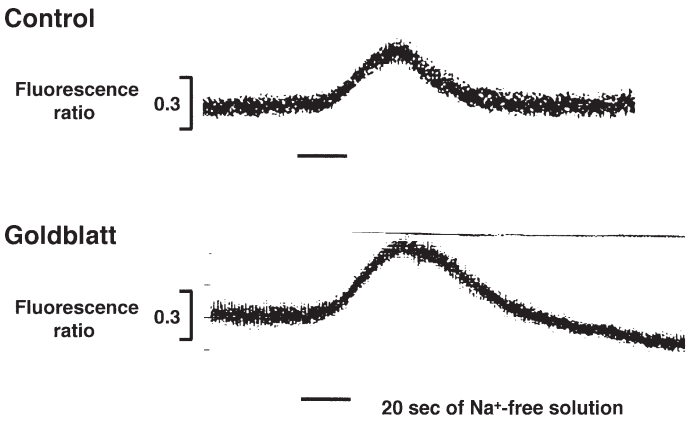

Fig. 5. Original traces of changes in intracellular Ca^{2+} during Na^+-free perfusion in sham-operated control rats (upper) and Goldblatt rats (lower) in constricted scale. The hearts were pretreated with 1 μM ryanodine. Goldblatt rats showed a slight prolongation of the time of Ca^{2+} increase and Ca^{2+} decrease as compared with control rats.

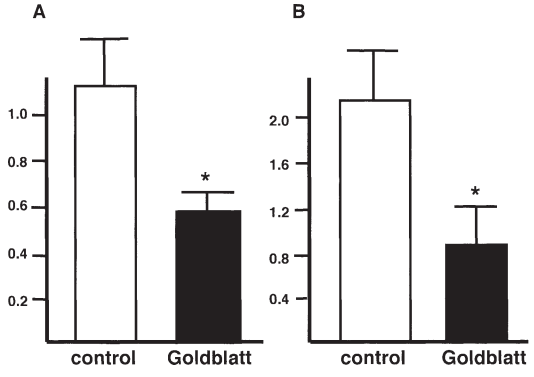

Fig. 6. Rate of Ca^{2+} amplitude to time-to-peak diastolic Ca^{2+} elevation (A) and rate of Ca^{2+} amplitude to 50% of declining time from peak to baseline Ca^{2+} (B) during Na^+-free perfusion after administration of 1 μM ryanodine, $^*p<0.05$ vs. control rats. $n=5$ for each group.

3. Effect of Na^+-free perfusion under ryanodine administration

Ten minutes of treatment with ryanodine markedly reduced the amplitude of the Ca^{2+} transient and increased the diastolic Ca^{2+} level. After a steady state was reached by means of perfusion for 15 minutes, the perfusate was replaced with a Na^+-free Tris solution for 20 seconds. The Tris solution was then replaced with standard perfusate again. Figure 5 shows a recording of this procedure. We found no significant differences in the time-to-peak or the 50% of declining time between the groups. However, the mean rate of Ca^{2+} increase was significantly lower in the Goldblatt rats (0.58 ± 0.11) than in control rats (1.19 ± 0.33, $p<0.05$; Fig. 6). The rate of Ca^{2+} decrease was also lower in Goldblatt rats (0.85 ± 0.40) than in control rats (2.18 ± 0.68; $p<0.05$, Fig. 6).

4. mRNA expression of the NCX

The mRNA expression of NCX, as measured with the RT-PCR method, decreased in Goldblatt rats (Fig. 7). Moreover, after mRNA expression was quantified, a comparison of these values using GAPDH as an internal control suggested a similar tendency to decrease (Fig. 8).

DISCUSSION

In this experiment, we used whole hearts with moderate-to-severe hypertrophy from Goldblatt rats as a model of renovascular hypertension. We found 1) decreases in the velocities of intracellular Ca^{2+} influx and efflux, 2) a decrease in NCX activity, and 3) a decrease in the mRNA expression of NCX. To our knowledge, no previous studies have examined these phenomena in Goldblatt rats in the same disease stage.

1. Experimental model

In this experimental model it is important to confirm whether fura-2 fluorescence accurately reflects the intracellular Ca^{2+} level. The fura-2 signals

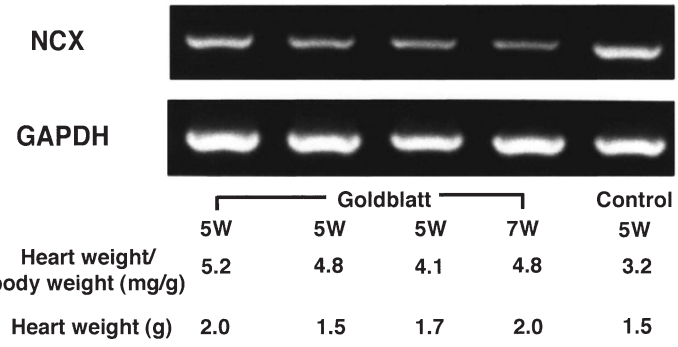

Fig. 7. Banding of the NCX and GAPDH using the RT-PCR reaction method. Compared with sham-operated control rats (similar results were observed in 3 animals, results of only one rat are shown), NCX mRNA expression in Goldblatt rat heart decreased ($n=4$).

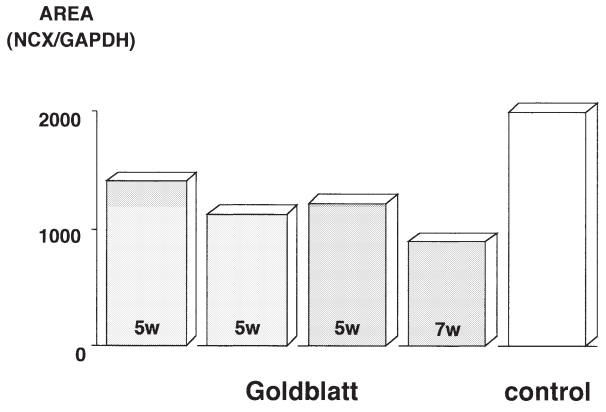

Fig. 8. NCX mRNA expression was corrected with GAPDH and imaging software.

may come from the cytoplasm and other areas. For example, Field et al. and Lee et al. have reported that mitochondria and endothelial cells contribute 30% to 40% of total fluorescence[25,26], but these sources have little effect on the characteristics of the signal in this model. Because the changes in intracellular Ca^{2+} concentration of endothelial cells or smooth muscle cells during contraction and relaxation of the heart were minimal, the form of the Ca^{2+} transient is not affected by the fluorescence from these cells. Therefore, this fluorescence indicator accurately reflects the intracellular Ca^{2+} concentration in the beating heart. This method, although technically complex, enables us to observe simultaneously intracellular Ca^{2+} and left ventricular pressure. Although some previous experiments have used isolated cardiomyocytes to evaluate Ca^{2+} influx or efflux and the function of NCX[9,27-29], few have used the whole heart[20,30].

2. Stage of hypertrophy in the heart

Cardiac hypertrophy is classified by severity as mild, moderate, or severe hypertrophy, which are known as compensatory stages. They are followed by the decompensatory stage, ultimately leading to heart failure. In this study, no clinical signs of heart failure, such as pulmonary edema, ascites, and congestive liver, were observed, nor were any clinical symptoms, such as dyspnea. However, the ratio of heart weight to body weight increased markedly as compared with that of rats used in previous reports. Therefore, the model in this study was compensatory but moderate-to-severe cardiac hypertrophy.

3. Changes in Ca^{2+} influx and efflux in the hypertrophied heart

Our results indicate that transient depletion of extracellular Na^+ causes the influx and efflux of Ca^{2+} and that these fluxes are slowed in the hypertrophic heart. Because we did not evaluate the Ca^{2+} transient and ventricular pressure in a single cardiac cycle, it is difficult to precisely compare the Ca^{2+} dynamics in our study with that in other experiments using single-cell preparations. However, our results obtained from whole hearts are similar to those of previous experiments using papillary muscle in which prolonged contraction and relaxation times were observed in association with slowing of the influx and efflux of Ca^{2+} [29]. We have also obtained similar results using Dahl salt-sensitive hypertensive rats[20].

4. Assessment of NCX in the hypertrophic heart

In this study, we used ryanodine for functional ablation of the SR. Activity of the NCX, as indicated by the velocity of Ca^{2+} flux, was selectively evaluated by adding ryanodine during Na^{+}-free perfusion. Both the forward and the reverse modes of NCX activities decreased in the hypertrophic whole-heart model. The activity of the NCX has previously been shown to decrease in the hypertrophic heart[27,28]. A recent report, however, has found that NCX activity does not change. We have previously reported that Ca^{2+} influx and efflux are slowed in association with a decrease in SERCA2a protein in Dahl rats with late-phase hypertrophic hearts. A consensus on this topic has not been achieved.

We suggest two possible reasons for this difference in reported NCX activity. The first possible reason is that NCX activity changes over time according to the degree of cardiac hypertrophy. This idea is based on a report showing that SERCA2a, which also regulates cytoplasmic Ca^{2+}, changes with the degree of hypertrophy[8]. Thus, the NCX, which similarly regulates cytoplasmic Ca^{2+}, may work reciprocally. The second possible reason is a difference in these animal models of the hypertrophic heart. In contrast to the spontaneously hypertensive rat and aortic constriction models, the renal artery constriction model has a markedly accelerated renin-angiotensin-aldosterone system, which is a circulatory endocrinological factor[31].

The mechanism of the link between the functions of SERCA2a and NCX is complicated. A recent study has found a decrease in NCX activity that is partially reversed by calcineurin inhibitors in rat hypertrophied cardiomyocytes subjected to chronic phenylephrine treatment[32]. The aortic-banded hypertrophic heart of heterozygous NCX knockout mice show better systolic function than do wild-type mice[33]. In the stage of hypertrophy, decreased NCX activity may play important roles in the elevation of cytoplasmic Ca^{2+}, the preservation of function, and the development of hypertrophy.

5. Changes in mRNA expression levels of the NCX in the hypertrophic heart

A major objective of this study was to examine the relationship between functional activity and mRNA expression levels of the NCX at the same stage of hypertrophy. Our results suggest that mRNA expression of the NCX tends to decrease in the hypertrophic heart. However, there have been a numerous reports concerning NCX mRNA expression. Ramírez-Gil et al. have reported that cardiac NCX mRNA expression decreases in a Guinea pig nephrectomy model[34]. Reinecke et al., however, have reported that phenylephrine administration increases NCX mRNA expression in hypertrophic rats hearts[35]. The difference might be due to different disease stages or to different models of hypertrophy. In an aortic-banding model, NCX activity did not change, although the NCX mRNA expression decreased[9]. In our experiments, both NCX activity and mRNA expression decreased, suggesting that the two are related. Because analysis of mRNA expression using the RT-PCR method, quantitative PCR, or Northern blotting might have produced different results, further investigations and quantification are needed. However, the banding images of the NCX obtained with the RT-PCR method are not saturated, whereas the results obtained with the PCR method seem to reflect the difference in mRNA expression.

The NCX activity and gene expression might be continuously altered in the transition from cardiac hypertrophy to heart failure. The present results partially support the notion of a compensatory relationship between NCX and SERCA2a. Future studies will need to examine the correlation between mRNA expression and the functional activity of the NCX and SERCA2a at different stages of cardiac hypertrophy. Furthermore, it is important to produce other animal models of the same stage of cardiac hypertrophy so that results can be compared.

CONCLUSION

In the whole hearts of Goldblatt rats with renovascular hypertension, simultaneous decreases in NCX functional activity and mRNA expression levels

were observed.

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