Effects of induced Na^+/Ca^{2+} exchanger overexpression on the spatial distribution of L-type Ca^{2+} channels and junctophilin-2 in pressure-overloaded hearts

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Abbreviations: NCX1, Na^+/Ca^{2+} exchanger 1; E-C coupling, excitation-contraction coupling; HF, heart failure; T-tubules, transverse-tubules; SR, sarcoplasmic reticulum; LTCC, L-type Ca^{2+} channel; JP2, junctophilin-2; TAC, transverse aortic constriction; DOX, doxycycline; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline containing

0.1% Tween 20; NA, numerical aperture; PIP_2, phosphatidylinositol 4,5-bisphosphate; Ank, Ankyrin; NKA, Na^+/Ca^{2+} exchanger 1 Na/K ATPase; IP_3R, inositol 1,4,5-trophosphate receptor

Abstract

The Na^+/Ca^{2+} exchanger 1 (NCX1) is an essential Ca^{2+} efflux system in cardiomyocytes. Although NCX1 is distributed throughout the sarcolemma, a subpopulation of NCX1 is localized to transverse (T)-tubules. There is growing evidence that T-tubule disorganization is a causal event that shifts the transition from hypertrophy to heart failure (HF). However, the detailed molecular mechanisms have not been clarified. Previously, we showed that induced NCX1 expression in pressure-overloaded hearts attenuates defective excitation-contraction coupling and HF progression. Here, we examined the effects of induced NCX1 overexpression on the spatial distribution of L-type Ca^{2+} channels (LTCCs) and junctophilin-2 (JP2), a structural protein that connects the T-tubule and sarcoplasmic reticulum membrane, in pressure-overloaded hearts. Quantitative analysis showed that the regularity of NCX1 localization was significantly decreased at 8 weeks after transverse aortic constriction (TAC)-surgery; however, T-tubule organization and the regularities of LTCC and JP2 immunofluorescent signals were maintained at this time point. These observations demonstrated that release of NCX1 from the T-tubule area occurred before the onset of T-tubule disorganization and LTCC and JP2 mislocalization. Moreover, induced NCX1 overexpression at 8 weeks post-TAC not only recovered NCX1 regularity but also prevented the decrease in LTCC and JP2 regularities at 16 weeks post-TAC. These results suggested that NCX1 may play an important role in the proper spatial distribution of LTCC and JP2 in

T-tubules in the context of pressure-overloading.

Key words: Na^+/Ca^{2+} exchanger 1, transverse-tubule, junctophilin-2, L-type Ca^{2+} channel, pressure-overloading, heart failure

1. Introduction

The Na^+/Ca^{2+} exchanger 1 (NCX1) plays an essential role in regulating Ca^{2+} homeostasis in cardiomyocytes. Under physiological conditions, NCX1 mainly extrudes Ca^{2+} into extracellular space during excitation-contraction (E-C) coupling and plays important roles in determining resting Ca^{2+} concentrations. However, the importance of NCX1 in the development of heart failure (HF) is unclear. Recently, we demonstrated that induced NCX1 overexpression attenuates pressure-overload-induced pathological cardiac remodeling [1]. Thus, maintaining NCX1 activity may be a potential therapeutic strategy for preventing the progression of HF.

Transverse (T)-tubules are invaginations of the surface sarcolemma with regular spacing along the longitudinal axis of myocytes. The highly organized T-tubule network physically and tightly couples with the terminal cisternae of the sarcoplasmic reticulum (SR), called dyads, throughout the entire myocyte. These structural couplings enable close contacts between voltage L-type Ca^{2+} channels (LTCC), which are mainly located on the T-tubule membrane, and Ca^{2+} release channels/ryanodine receptors on the SR, guaranteeing the instantaneous excitation and synchronous triggering of SR Ca^{2+} release during E-C coupling [2]. T-tubule disorganization is tightly linked to impaired Ca^{2+} handling and contractility and represents a common pathological alternation in failing myocytes [2-4]. Interestingly, induced NCX1 overexpression also prevents T-tubule disorganization in pressure-overloaded hearts

[1]. However, the role of NCX1 in the maintenance of T-tubule structure has not yet been elucidated.

One of the most extensively studied molecules influencing the T-tubule structure is junctophilin-2 (JP2). JP2 is a membrane-binding protein that physically connects the T-tubule membrane and SR membrane [5]. We and others have reported that T-tubule disorganization is associated with a significant decrease in JP2 expression [1, 6, 7]. In addition, JP2 knockdown results in T-tubule disorganization [6] and reduced T-tubule maturation [8]. In contrast, overexpression of JP2 prevents T-tubule disorganization in pressure-overloaded hearts [9]. Although these results strongly suggest that JP2 is important for maintaining the T-tubule structure, our knowledge of the molecular mechanisms underlying the downregulation of JP2 in failing hearts is limited. Notably, however, experimental results have shown that proteolytic cleavage of JP2 is dependent on Ca^{2+} [10], suggesting that JP2 downregulation may be associated with Ca^{2+} dysregulation in the microdomain of the cardiac dyad.

Accordingly, in this study, we aimed to elucidate the role of NCX1 in the maintenance of the T-tubule structure during the development of HF. To this end, we examined the effects of induced NCX1 overexpression on the spatial distribution of LTCCs and JP2 at T-tubules. We evaluated changes in the localization of NCX1, LTCCs, and JP2 during pressure-overload-induced HF after transverse aortic constriction (TAC) surgery and

quantitatively evaluated the patterns of NCX1, LTCC, and JP2 localization by fast Fourier transform (FFT) processing of immunofluorescent images. Finally, we investigated the role of NCX1 in maintaining the spatial distribution of LTCCs and JP2 in pressure-overloaded hearts of transgenic mice showing cardiac-specific, doxycycline (DOX)-dependent NCX1 expression.

2. Materials and Methods

2.1 Animals

The Institutional Animal Care and Use Committees at Okayama University and Kawasaki Medical School approved the animal experiments conducted in this study. All methods were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

To control expression level of NCX1 in cardiomyocytes, we used transgenic mice in which NCX1 expression was controlled by a cardiac-specific, DOX-dependent promoter, as previously described [1]. We crossed two lines of transgenic mice: one carrying the canine *NCX1* gene under the control of the minimal cytomegalovirus promoter, and the other carrying a gene encoding the reverse tetracycline transactivator (rtTA) protein under the control of an α-myosin heavy chain promoter. DOX (1 mg/day/mouse; Clontech, CA, USA) was administered intraperitoneally beginning 8 weeks after TAC surgery.

2.2 TAC surgery

Ten-week-old male mice were subjected to left ventricular pressure-overload by TAC surgery, as described previously [1]. Mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine and ventilated with a respirator (SN-480-7; Shinano, Tokyo, Japan). A small incision through the second intercostal space was created, and the transverse aorta was constricted with 7-0 nylon string by ligating the aorta with a blunted 27-gauge needle, which was removed before the chest wall was closed.

2.3 Fluorescent imaging

Immunostaining of mouse hearts was performed as described previously [1, 11]. Removed hearts were embedded in OCT compound (Tissue-Tek Cryomold, Sakura Finetek, Tokyo, Japan) and were frozen. Frozen heart blocks were cut into 5-μm-thick sections. These sections were air-dried, washed with phosphate-buffered saline (PBS), and permeabilized with 0.1% Triton X-100 for 5 min. Next, the sections were incubated in blocking solution for 1 h and then with polyclonal antibodies against NCX1 (generated in our laboratory [12]; 1:1000 dilution), LTCC (1:1000 dilution; Alomone labs, Jerusalem, Israel), or JP2 (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibodies for 1 h at 4°C. The sections were then washed with PBS containing 0.1% Tween 20 (PBS-T) and

incubated with Alexa Flour 488-conjugated anti-rabbit IgG (A11008; Life Technologies, CA, USA) or Alexa Flour 568-conjugated anti-Goat IgG (A-11057; Life Technologies) as secondary antibodies for 15 min at 4°C. The sections were washed with PBS-T and incubated with 4′ ,6-diamidino-2-phenylindole (DAPI; Life Technologies) in PBS-T for 5 min at 4°C to stain nuclei. The sections were rinsed with PBS-T again and mounted with Dako fluorescent mounting medium (Dako North America, CA, USA). Fluorescent images were obtained using a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan) equipped with an UPlanApo 60× (numerical aperture [NA] = 1.35) oil immersion objective lens (Olympus).

To visualize T-tubules in cardiomyocytes, isolated cells were incubated with 10 μM di-8-ANEPPS (Molecular Probes, Eugene, OR, USA) for 10 min at room temperature, as described previously [1]. Images were obtained with a confocal system, as described above.

2.4 Image analysis

To quantitatively evaluate the regularities of LTCC, JP2, and NCX1 localization, we obtained the one-dimensional power spectra of LTCC, JP2, and NCX1 immunofluorescent signals using FFT, as computed by the FFT tool in Origin Pro 9.0.0J (OriginLab Corporation, USA), as previously described [1]. For FFT analysis, immunofluorescence images were converted to binary images using ImageJ 1.46r software (National Institutes of Health, USA),

and an intensity profile was obtained randomly along the longitudinal axis of the cardiomyocyte, avoiding the nucleus. The power-spatial frequency relationship was calculated, and the peak amplitude was taken as an index of the regularity.

2.5 Statistical analysis

Results are expressed as means ± standard errors of the means (SEMs). For multiple comparisons, analysis of variance with Tukey-Kramer tests was performed. Differences with *p* values of less than 0.05 were considered statistically significant.

3. Results

In a previous study, we demonstrated that NCX1 overexpression prevented T-tubule disorganization in pressure-overload hearts [1]. Consistent with this previous study, we found a striated staining pattern using the membrane-binding dye di-8-ANEPPS, demonstrating that the T-tubule structure was disordered at 8–16 weeks after TAC. Notably, this pattern was maintained by NCX1 overexpression, induced via DOX treatment from 8 weeks after TAC operation (Fig. 1).

Next, we analyzed the spatial distributions of LTCC, JP2, and NCX1 in TAC hearts (Fig. 1B). Before TAC, LTCC, JP2, and NCX1 were localized in T-tubules, and the immunofluorescent signals appeared as a well-ordered pattern (Fig. 1B). The staining

patterns of LTCC and JP2 signals were still maintained at 8 weeks after TAC. However, the pattern appeared to be disordered at 12 weeks after TAC and further disrupted at 16 weeks after TAC (Fig. 1B). In contrast, NCX1 signals already showed an irregular pattern at 8 weeks after TAC and then exhibited reduced intensity of immunoreactivity at 16 weeks after TAC (Fig. 1B).

To quantitatively evaluate changes in the localizations of LTCC, JP2, and NCX1 with HF progression, the power spectra of their immunofluorescent signals were analyzed by FFT (Fig. 1C). The dominant peaks occurred at approximately 0.5 μm^{-1}, corresponding to T-tubule spacing (approximately 2 μm). We used the peak power value as a quantitative index of regularity (Fig. 1D). While regularities of LTCC and JP2 significantly decreased at 12 and 16 weeks after TAC, they were maintained until 8 weeks after TAC (Fig. 1C and 1D). Thus, the spatial distributions of LTCC and JP2 were consistent with remodeling of the T-tubule structure in TAC hearts (Fig. 1A). In contrast, NCX1 regularity was significantly decreased at 8 weeks after TAC, indicating that the release of NCX1 protein from the T-tubule occurred before the onset of disorganization of the T-tubule structure (Fig. 1C and 1D). These results suggested that disordered NCX1 localization triggered the disruption of LTCC and JP2 localization at T-tubules.

To elucidate the importance of NCX1 for LTCC and JP2 localization at T-tubules, we injected transgenic mice with DOX beginning at 8 weeks after TAC to induce NCX1

overexpression (Supplemental Fig. 1). Induced NCX1 expression recovered NCX1 regularity to the pre-TAC level at 4 weeks after DOX injection (12 weeks post-TAC), and this recovered NCX1 regularity was sustained for up to 16 weeks after TAC (Fig. 2A and 2B, Supplemental Fig. 2). In addition, induced NCX1 expression for 4 weeks prevented the degradation of JP2 and LTCC regularities (Fig. 2A and 2B, and Supplemental Fig. 2). JP2 and LTCC regularities were recovered to the pre-TAC level at 8 weeks after initiation of DOX administration (i.e., 16 weeks post-TAC). Thus, the spatial distributions of JP2 and LTCC depended on NCX1 localization. These results suggested that induced NCX1 overexpression contributed to maintenance of JP2 and LTCC localization at the T-tubule membrane under prolonged pressure-overloading.

To gain deeper insights into the effects of induced NCX1 overexpression on JP2 and LTCC localization at the T-tubule membrane, we plotted the relationships between the regularities of JP2 and LTCC and that of NCX1 during pressure-overload-induced HF progression with or without DOX treatment (Fig. 3). The relationships between the regularities of JP2 and LTCC were highly linear during progression of HF and NCX1 overexpression (Fig. 3). JP2 regularity tended to vary linearly with NCX1 regularity (Fig. 3 middle panel). However, the relationships between JP2 and NCX1 regularities at 8 weeks after TAC and 4 weeks after DOX injection (12 weeks post-TAC) were different from those at other time points. The relationship at 8 weeks post-TAC shifted leftward, suggesting that a

decrease in NCX1 regularity occurred before the decrease in JP2 regularity. In contrast, the relationship at 4 weeks after DOX injection (12 weeks post-TAC) shifted rightward, showing that an increase in NCX1 regularity occurred prior to the increase in JP2 regularity. Changes in the relationships between LTCC and NCX1 showed the same tendency as those between JP2 and NCX1 (Fig. 3). Collectively, these findings demonstrated that NCX1 regularity was decreased before JP2 and LTCC regularities and that NCX1 regularity was recovered by DOX injection before JP2 and LTCC regularities. These results suggested that NCX1 played an important role in the maintenance of JP2 and LTCC localization at T-tubules.

4. Discussion

In the present study, we quantitatively evaluated changes in patterns of NCX1, JP2, and LTCC localization during progression of HF induced by prolonged pressure-overload. Our results revealed that NCX1 localization was disordered before the onset of T-tubule disorganization and LTCC and JP2 mislocalization. We also found that induced NCX1 overexpression in pressure-overload hearts facilitated the recovery of NCX1, LTCC, and JP2 localizations to pre-TAC levels. These findings demonstrated that the localization of LTCC and JP2 depended on NCX1 distribution during HF progression, suggesting that NCX1 may have a pivotal role in the maintenance of LTCC and JP2 localization at T-tubules.

One of the main findings in this study was that the release of NCX1 protein from

T-tubules and the sarcolemma occurred before the onset of LTCC and JP2 mislocalization at 8 weeks post-TAC. Because the T-tubule structure was maintained at this stage, it is likely that NCX1 may have been selectively internalized in the hearts. Because the endocytosis machinery is activated in hypertrophic cardiomyocytes [13], cargo-dependent endocytosis involving depressed NCX1 activity at the T-tubule membrane and sarcolemma may also be enhanced in hearts at 8 weeks post-TAC. A recent study reported that palmitoylation of NCX1 at a single cysteine in its large intracellular loop controls its inactivation and internalization during stress signaling [14]. Palmitoylated NCX1 has also been found in cardiac muscle and is involved in Ca^{2+} homeostasis [14]. In our study, palmitoylated NCX1 may have accelerated internalization and showed reduced Ca^{2+} extrusion activity in the microdomain in hearts at 8 weeks post-TAC. Additionally, in our previous study, we showed that intracellular Na^{+}-dependent Ca^{2+} uptake into cells was decreased in hearts at 8 weeks post-TAC [1]. Therefore, further studies are needed to determine whether reduced NCX1 activity may be associated with palmitoylation.

In addition, previous data have shown that NCX1 internalization is promoted by phosphatidylinositol 4,5-bisphosphate (PIP_2) synthesis, high cytosolic Na^{+}, activation of Gq-coupled receptors, and metabolic stress [15]. Additionally, we reported that PIP_2 accumulation in the T-tubule and sarcolemma is increased at 8 weeks after TAC [1]. Because the changes in biochemical properties during HF progression are multifaceted and complex,

other mechanisms may also mediate the internalization of NCX1 in TAC hearts.

The mislocalization of NCX1 also occurs in ankyrin (Ank)-B $^{+/-}$ cardiomyocytes [16]. Ank-B regulates cardiac Ca^{2+} and contractility by controlling the proper targeting and retention of the NCX1 Na/K ATPase (NKA), inositol 1,4,5-trophosphate receptor (IP_3R), and protein phosphatase 2A [16, 17]. In contrast, the localization of LTCCs is independent of Ank-B expression [16]. Therefore, the Ank-B-dependent complex of NCX1, NKA, and IP_3R at T-tubule/SR sites may be distinct from the classic 'dyad'. Thus, although NCX1 is largely localized at the outside of dyads, the functional Ca^{2+} microdomain could extend beyond the dyad [18, 19]. Accordingly, NCX1 may contribute to the maintenance of low Ca^{2+} concentrations in the microdomain of the dyad.

In failing myocytes, Ca^{2+}-dependent proteolysis of JP2 by calpain activation is accelerated and causes T-tubule disorganization [20, 21]. In this study, the mislocalization of JP2 occurred later than NCX1 release from the membrane in hearts at 12 weeks post-TAC, suggesting that increased Ca^{2+} concentrations in the microdomain caused by depressed NCX1 function may trigger the degradation of JP2. Previous studies have reported that knockdown of JP2 disrupts T-tubule organization [6] and that overexpression of JP2 protects against T-tubule disorganization after TAC [9]. These observations suggest that the T-tubule structure may depend on JP2 localization. Thus, stabilizing JP2 by controlling local Ca^{2+} concentrations in the microdomain may lead facilitate the maintenance of the T-tubule

structure, in which NCX1 has a pivotal role.

A recent study demonstrated the intracellular localization of LTCCs in human HF [22]. Although the detailed molecular mechanisms of LTCC internalization in cardiomyocytes have not yet been clarified, evidence suggests that depolarization induces Ca^{2+}-dependent internalization of LTCCs in neurons [23]. In hearts, reduced expression of the membrane anchor amphyphisin-2/Bin1 not only enhances LTCC internalization but also contributes to disease-related T-tubule disorganization [22, 24, 25]. In our study, decreased LTCC regularity was observed in hearts at 12 weeks post-TAC. Because reduced JP2 regularity also occurred during this time, the intracellular localization of LTCCs may be explained by the influence of T-tubule disorganization. Moreover, release of NCX1 from T-tubules in hearts at 8 weeks post-TAC may affect LTCC internalization in hearts at 12–16 weeks post-TAC. Similarly, the deletion of NCX1 from mouse hearts leads to a compensatory reduction of nearly 60% in the LTCC current [26], while increased NCX1 activity in the heart due to transgene-mediated overexpression produces more LTCC current [27]. Thus, there is a functional coupling between NCX1 and LTCCs at T-tubules via a Ca^{2+}-dependent mechanism.

Another interesting finding in this study is that induced NCX1 overexpression after 8 weeks post-TAC not only recovered NCX1 regularity but also prevented the decrease in LTCC and JP2 regularities at 16 weeks post-TAC. These observations emphasized the essential role of NCX1 in LTCC and JP2 localization at the T-tubule and highlighted this

factor as a regulatory of Ca^{2+} at the microdomain for maintenance of the T-tubule structure in pressure-overload-induced HF. Future studies are needed to clarify the role of NCX1 in the maintenance of the T-tubule structure.

In conclusion, our current findings demonstrated that T-tubule structures and LTCC and JP2 localization depended on the NCX1 distribution during the development of hypertrophy and progression of HF induced by pressure-overloading. These findings provide novel insights into the roles of NCX1 in failing myocytes and show that the maintenance of NCX1 may represent a new strategy for blocking HF progression.

Conflict of Interest

None.

Acknowledgements

This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (grant nos. JP26282122 and JP26560211 to Y.K., JP26702014 to Y.U., and JP26282127 to S.M.), the Japan Society for the Promotion of Science (Funding Program for Next Generation World-Leading Researchers [NEXT Program] to Y.K.), and the Japan Agency for Medical Research and Development (AMED PRIME to Y.K.).

Figure legends

Fig. 1. Changes in the localization patterns of LTCC, JP2, and NCX1 during HF progression. (A) Representative confocal images of cardiomyocyte membrane staining by di-8-ANEPPS. Cardiomyocytes were isolated from hearts prior to TAC, at 8 and 16 weeks post-TAC (TAC8w and TAC16w), and at 16 weeks post-TAC with injection of DOX at 8 weeks post-TAC [TAC16w(+DOX8w)]. Scale bar, 50 μm; inset scale bar, 5 μm. (B) Representative confocal images of myocytes stained for LTCC, JP2, and NCX1. Scale bar, 20 μm. (C) Representative power-spatial frequency relationships of LTCC, JP2, and NCX1. (D) Regularities of LTCC, JP2, and NCX1. Regularity was normalized to that prior to TAC. LTCC: n = 20, 18, 18, and 16; JP2: n = 26, 27, 21, and 29; NCX1: n = 41, 40, 31, and 31 cells for pre-TAC, TAC8w, TAC12w, and TAC16w, respectively. Data are means ± SEMs. *P < 0.05 versus pre-TAC; #P < 0.05 versus 8 weeks post-TAC.

Fig. 2. Induced NCX1 overexpression recovered the regularities of NCX1, JP2, and LTCC in pressure-overloaded hearts. (A) Representative confocal images of NCX1, JP2, and LTCC. Scale bar, 20 μm. (B) Regularities of NCX1, LTCC, and JP2. Regularity was normalized to that prior to TAC. NCX1: n = 31 and 30, LTCC: n = 19 and 13, JP2: n = 22 and 25 cells for TAC12w(+DOX4w) and TAC 16w(+DOX8w), respectively. Data are means ± SEMs. *P < 0.05 versus pre-TAC.

Fig. 3. Plots of changes in the relationships between LTCC, JP2, and NCX1 regularities in hearts pre-TAC; at 8, 12, and 16 weeks after TAC; and at 4 and 8 weeks after DOX injection (post-TAC 12 and 16 weeks). Data are means ± SEMs. Lines represent regression lines obtained from regularity relationships in hearts pre-TAC, at 12 and 16 weeks after TAC, and at 8 weeks after DOX injection (16 weeks post-TAC).

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