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Essential role of STIM1 in the development of cardiomyocyte hypertrophy

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ABSTRACT

Store-operated Ca^{2+} entry (SOCE) through transient receptor potential (TRP) channels is important in the development of cardiac hypertrophy. Recently, stromal interaction molecule 1 (STIM1) was identified as a key regulator of SOCE. In this study, we examined whether STIM1 is involved in the development of cardiomyocyte hypertrophy. RT-PCR showed that cultured rat cardiomyocytes constitutively expressed STIM1. Endothelin-1 (ET-1) treatment for 48 h enhanced TRPC1 expression, SOCE, and nuclear factor of activated T cells activation without upregulating STIM1. However, the knockdown of STIM1 suppressed these effects, thereby preventing a hypertrophic response. These results suggest that STIM1 plays an essential role in the development of cardiomyocyte hypertrophy.

Introduction

Sustained Ca^{2+} entry is required for the development of cardiomyocyte hypertrophy [1–4]. Transient receptor potential canonical (TRPC) channels form various Ca^{2+}-entry channels in the heart. Recent studies have shown that TRPC1, TRPC3, and/or TRPC6 play a crucial role in the development of cardiac hypertrophy [5–9]. We previously demonstrated that during the development of cardiac hypertrophy, the number of store-operated Ca^{2+} (SOC) channels containing TRPC1 increased and that these channels regulated the signaling pathways that govern cardiac hypertrophy [5–7]. However, the mechanism underlying the activation of TRPC1-containing SOC channels is unknown. Although the molecular identity and regulation of SOC channels have not been precisely determined [10], a notable recent finding is the identification of STIM1, which may regulate SOC channels as a Ca^{2+} sensor in the endoplasmic reticulum [11–14]. In T lymphocytes, STIM1 is involved in cytokine production through store-operated Ca^{2+} entry (SOCE) and subsequent nuclear factor of activated T cells (NFAT) activation. STIM1 contains 685 amino acids that comprise an N-terminal EF hand, a transmembrane domain, and two coiled-coil domains. The EF hand domain is predicted to function in Ca^{2+} binding. STIM1 is localized to the endoplasmic reticulum and plasma membrane, where its coiled-coil domains lie in the intracellular space [11,12,15,16]. These coiled-coil domains may mediate the interaction between STIM1 and various TRPC proteins. STIM1 interacts directly with TRPC1, TRPC2, TRPC4, and TRPC5 but not with TRPC3, TRPC6, or TRPC7 [16]. Previously, we reported that STIM1 together with TRPC1 was essential for SOCE and that STIM1 was involved in vascular smooth muscle cell hypertrophy and proliferation [17,18]. In the present study, we tested whether STIM1 is involved in the development of rat cardiomyocyte hypertrophy through the regulation of TRPC1-mediated SOCE.

Materials and methods

Cell culture. Cardiomyocytes from 2-day-old Wistar rats were isolated and cultured as described previously [7]. The cardiomyocytes were analyzed using inverted confocal laser-scanning microscopy (Zeiss LSM510META, Carl Zeiss, Oberkochen, Germany). The surface area of the cells was determined using the program LSM Image Browser.

RNA extraction and RT-PCR. Two micrograms of template were reverse-transcribed using oligo (dT) primer in a final volume of 20 μl. Rat *STIM1* and *TRPC1* were then amplified. The sequences of the primers are given in the supplemental information. Comparative RT-PCR was performed under the same conditions with 30 cycles, except for the reactions involving BNP and TRPC1, which contained 25 cycles.

Abdominal aortic banding (*AAB*). AAB was performed as described previously [7]. Sham-operated animals ($n = 11$) were subjected to the same procedure without aortic banding. The hearts were isolated for four weeks after the operation. The Animal Ethics Committee of the Akita University School of Medicine approved the study protocol.

Western blotting. All protein samples were purified from the microsomal fraction, which is composed mainly of plasma mem-

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brane, by ultracentrifugation (105,000*g* for 60 min). To test for STIM1, TRPC1, TRPC5, and TRPC6 (Alomone Labs, Jerusalem, Israel), we subjected each sample (50 μg) to 6% SDS–PAGE, transferred it to a PVDF membrane, and blocked it by rocking for 1 h at 4 °C in blocking buffer (0.1% Tween 20 and 1% bovine serum albumin in Tris-buffered saline). An ECL detection system was used to detect the bound antibody. An anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as an internal loading control.

siRNA knockdown of STIM1. Cells were seeded onto 35-mm dishes the day before transfection. Transfection of the double-stranded RNA (50 nM final concentration) was performed according to the manufacturer's protocol for HiPerFect Transfection Reagent (Qiagen Japan, Tokyo, Japan). STIM1 siRNA (target CAGCTTTGAGGCCGTCCGCAA) and a negative control siRNA (target AATTCTCCGAACGTGTCACGT) for mock transfection were obtained from Qiagen Japan.

Measurement of the intracellular calcium concentration ($[Ca^{2+}]_i$). Thapsigargin (TG, 1 μM)-stimulated SOCE in response to a change from Ca^{2+}-free conditions to 5 mM Ca^{2+} was measured in the presence of 10 μM verapamil as described previously [5,19].

NFAT promoter activity assay. An NFAT-GFP reporter plasmid (Stratagene, Cedar Creek, TX, USA) was transfected into rat cardiomyocytes using Lipofectamine 2000™ reagent (Invitrogen Japan) according to the manufacturer's instructions. After a 48-h incubation, the cells were analyzed using confocal laser-scanning microscopy.

Statistical analysis. The data are presented as the mean ± SE. Differences were evaluated using unpaired Student's *t*-tests; $p < 0.01$ was considered statistically significant.

Results

Detection of STIM1 in neonatal rat cardiomyocytes and adult rat hearts

We used RT-PCR to investigate the expression of STIM1 in neonatal rat cardiomyocytes (Fig. 1A). The rat cardiomyocytes constitutively expressed STIM1 as well as TRPC1. To examine the enhanced expression of STIM1 in hypertrophied heart, we analyzed the expression of STIM1 in the hearts of abdominal aortic–banded rats, a typical animal model of cardiac hypertrophy (heart weight/body weight ratio: sham, 2.40 ± 0.05; AAB, 2.89 ± 0.09; $p < 0.01$, $n = 11$) [7]. Western blot analysis revealed constitutive STIM1 expression in adult rat heart (Fig. 1B). However, only marginal changes in STIM1 were detected in hypertrophied heart compared with normal heart. Consistent with our previous data, TRPC1 expression was significantly increased in rats subjected to AAB (259 ± 36%, $n = 3$), whereas TRPC5 and TRPC6 expression was unchanged (Fig. 1C). Anti-GAPDH antibodies were used to verify that comparable amounts of protein were loaded in each lane of the gel.

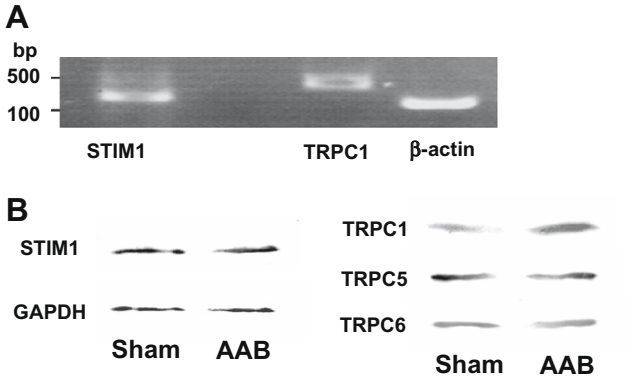

Fig. 1. (A) Examination of STIM1, TRPC1, and β-actin expression by RT-PCR. (B) Immunoblotting for STIM1 and TRPCs in hypertrophied heart of abdominal aortic–banded rat. Each experiment was repeated three times with the same results.

Effects of STIM1 on SOCE and NFAT activation

To examine the role of STIM1 in cardiomyocytes, we examined SOCE in cardiomyocytes, because STIM1 is a key regulator of SOC channels. Changes in the $[Ca^{2+}]_i$ in individual cells were measured based on the fluorescence ratio of Fura-2AM in the presence of 10 μM verapamil. SOCE was activated by the depletion of intracellular Ca^{2+} stores using the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor TG (1 μM) in the absence of extracellular Ca^{2+} followed by the restoration of 5 mM extracellular Ca^{2+} (Fig. 2A). Enhanced SOCE was observed in the ET-1-treated group compared with the mock-transfected group (267 ± 6.7% increase vs. control, $p < 0.01$; $n = 39$, ET-1; $n = 51$, control). In contrast, SOCE was significantly decreased in the siSTIM1 + ET-1 group (83 ± 1.8% decrease vs. mock + ET-1-treated group, $p < 0.01$). Our results suggest that STIM1 is critically involved in SOCE in cardiomyocytes. To examine subsequent NFAT signaling as a downstream event of sustained Ca^{2+} entry in cardiomyocyte hypertrophy, we evaluated NFAT promoter activity based on the EGFP fluorescence intensity. Although ET-1 treatment led to increased NFAT-EGFP fluorescence (Fig. 2B), cardiomyocytes in which STIM1 was knocked down showed minimal NFAT activity, indicating the importance of STIM1 in eliciting NFAT activity. Overall, these data suggest the involvement of STIM1 in the regulation of SOCE and NFAT activity in cardiomyocytes.

Knockdown of STIM1 inhibits hypertrophic responses

The levels of STIM1, BNP, and β-actin are shown in Fig. 3A. The inhibitory effect of siRNA treatment on STIM1 expression became obvious 48 h after transfection. Cardiomyocytes were exposed to ET-1 (10 nM), AT II (100 nM), or PE (100 μM) for 48 h after siRNA transfection. Apparent changes in STIM1 were not detected in the mock + ET-1-, AT II-, or PE-treated groups compared with mock-transfected cardiomyocytes. However, the level of STIM1 in the siSTIM1 group was decreased to approximately 8% of the mock level (lane 8), suggesting the successful knockdown of STIM1. To address whether the knockdown of STIM1 attenuated the hypertrophic response, we examined the expression of BNP, a cardiac fetal gene as a marker of cardiac hypertrophy. RT-PCR analysis showed that the level of BNP in the mock + ET-1, AT II, and PE groups was increased (lanes 2, 4, and 6). In contrast, the knockdown of STIM1 inhibited the expected increases in BNP following 48 h of incubation with ET-1, AT II, or PE (lanes 3, 5, and 7). RT-PCR analysis revealed the comparable amplification of β-actin-specific bands by PCR, suggesting equal experimental conditions. The experiment was repeated five times with similar results. Moreover, we examined the cell surface area in each group. The surface area of the mock + ET-1-, AT II-, and PE-treated cardiomyocytes was significantly increased compared with the control group; however, the knockdown of STIM1 suppressed these agonist-induced increases in cell size (Fig. 3B). Taken together, these results clearly indicate that STIM1 plays an essential role in the hypertrophic response induced by ET-1, AT II, or PE.

We previously reported that the level of TRPC1 was closely related to the development of cardiac hypertrophy; thus, we examined the expression level of TRPC1. TRPC1 was upregulated in ET-1-treated cardiomyocytes (lane 2, 108 ± 4% increase vs. lane 1; Fig. 3B). In the STIM1 knocked-down cardiomyocytes, the upregulation of TRPC1 was prevented (lane 3, 46 ± 1% decrease vs. lane 2). The experiment was repeated three times with similar results.

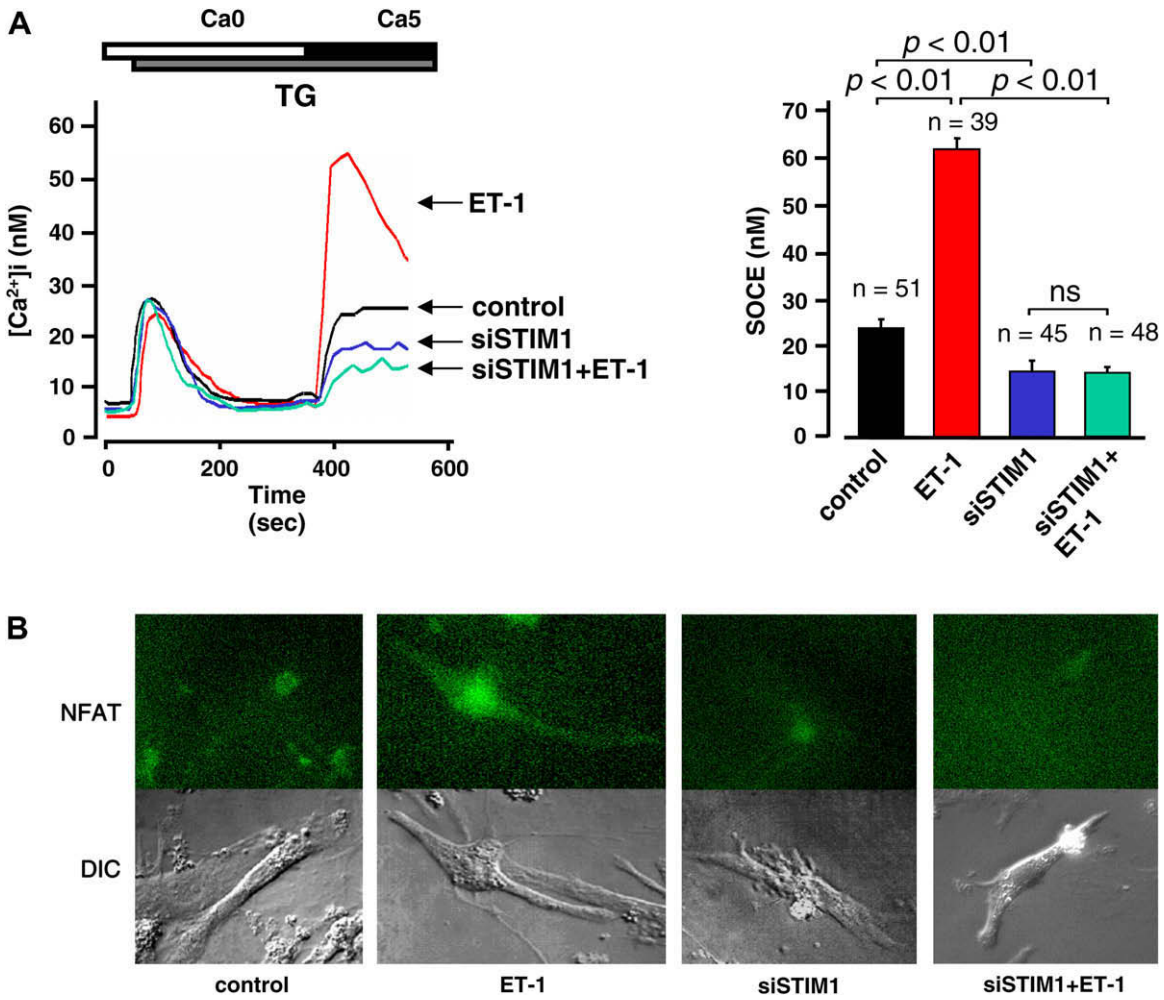

Fig. 2. (A) Representative records of SOCE in cardiomyocytes. The statistical data are presented as the means ± SE. (B) Representative EGFP fluorescence, indicating NFAT activation, and the corresponding differential interference contrast (DIC) images are shown.

Taken together, these results clearly indicate that STIM1 plays an essential role in the hypertrophic response through TRPC1.

Discussion

Several studies have suggested that Ca^{2+} handling plays an important role not only in cardiac contractility but also in cardiac hypertrophy [20]. Despite extensive investigations, the actual source and regulation of the Ca^{2+} involved in the development of cardiomyocyte hypertrophy has not been precisely determined. We previously showed that TRPC1 contributed to cardiac hypertrophy via SOCE and subsequent NFAT activation [7]. Here, we show that the knockdown of STIM1 inhibited the upregulation of TRPC1 and the hypertrophic response induced by ET-1, AT II, or PE. This is the first report to show a functional link between STIM1 and the signaling pathways that govern cardiac hypertrophy.

The molecular identity of SOC channels is poorly understood; however, TRPC1 is considered to be a pivotal component [21]. In mouse pulmonary artery smooth muscle cells, TRPC1 is directly activated by STIM1 [22]. Our knockdown assay showed that siSTIM1 inhibited SOCE and NFAT activation and prevented increases in BNP expression and cell size, suggesting that STIM1 is a critical factor in hypertrophic responses. Moreover, siSTIM1 inhibited the upregulation of TRPC1. Previously, we reported that TRPC1 upregulation was facilitated through a feed-forward mechanism, based on the fact that TRPC1 has an NFAT binding sequence in its promoter. It is likely that the knockdown of STIM1 halts this feed-forward mechanism and inhibits TRPC1 expression. STIM1 also contributes to the hypertrophic response through the regulation of TRPC1 expression. On the other hand, there were no differences in the STIM1 expression level between normal hearts and the hypertrophied hearts of abdominal aortic–banded rats. In cardiomyocytes, it is thought that protein level of the endogenous STIM1 is sufficient to facilitate hypertrophic responses without its upregulation.

A controversial issue is which TRPC molecule is most important. TRPC (TRPC1, TRPC3, and TRPC6) activation might have an important role in the signaling pathways that lead to cardiac hypertrophy [5–9]. It is interesting that a previous study showed that STIM1 binds to TRPC1, TRPC2, TRPC4, and TRPC5 but not to TRPC3, TRPC6, or TRPC7 [16]. Taken together, these data suggest that the STIM1–TRPC1 system is more important than the others for cardiac hypertrophy. However, our findings do not preclude a role for other TRPCs in mediating hypertrophic responses, because TRPC proteins are thought to form heteromeric Ca^{2+}-entry channels with different TRP subunits. Moreover, because TRPC3, TRPC6, and TRPC1 have conserved NFAT consensus sequences in their promoters, siSTIM1 may inhibit the expression of other TRPCs. As clinically important cardiac hypertrophy arises over a long period of time, the combination of TRPC channels might be altered during its development. Nevertheless, we propose the STIM1–TRPC1 system is more important than the others for cardiac hypertrophy.

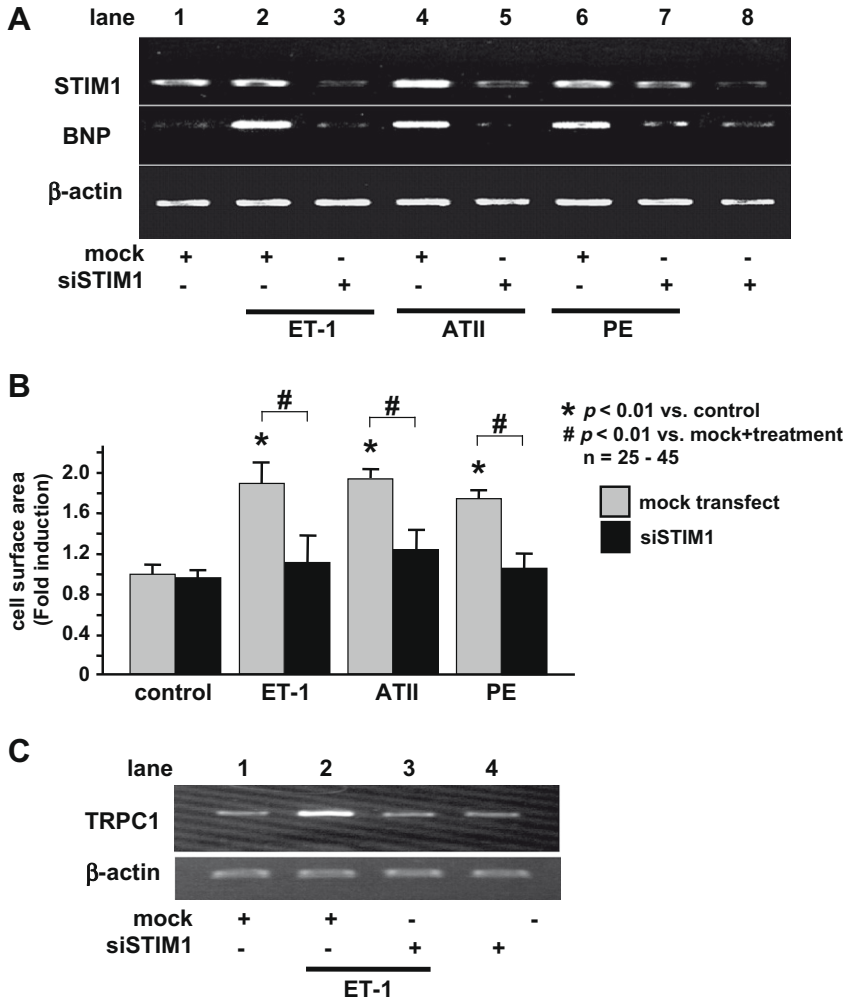

Fig. 3. (A) Comparative RT-PCR analysis of STIM1 and BNP expression was performed as indicated in the lower panel. (B) After treatment with ET-1 (10 nM), AT II (100 nM), or PE (100 μM) for 48 h after siRNA transfection, cell surface area was assessed. The results are presented as the means ± SE of 25–45 cells. $^{*}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. mock + treatment. (C) Comparative RT-PCR analysis of TRPC1 expression. The experiment was repeated five times with similar results; representative data are shown.

Current thinking is that SOC channels are regulated by STIM1 and require the translocation of STIM1 from the endoplasmic reticulum toward the plasma membrane in response to the depletion of Ca^{2+} from the endoplasmic reticulum; however, the actual mechanism of STIM1 translocation is unknown. Further study is needed to clarify whether STIM1 is translocated in response to ET-1 in cardiomyocytes.

In conclusion, STIM1 together with TRPC1 plays a pivotal role as a functional regulator of cardiac hypertrophy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.117.

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