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# Sildenafil prevents the up-regulation of transient receptor potential canonical channels in the development of cardiomyocyte hypertrophy

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ABSTRACT

*Background:* Transient receptor potential canonical (TRPCs) channels are up-regulated in the development of cardiac hypertrophy. Sildenafil inhibits TRPC6 activation and expression, leading to the prevention of cardiac hypertrophy. However, the effects of sildenafil on the expression of other TRPCs remain unknown. We hypothesized that in addition to its effects of TRPC6, sildenafil blocks the up-regulation of other TRPC channels to suppress cardiomyocyte hypertrophy.
*Methods and results:* In cultured neonatal rat cardiomyocytes, a 48 h treatment with 10 nM endothelin (ET)-1 induced hypertrophic responses characterized by nuclear factor of activated T cells activation and enhancement of brain natriuretic peptide expression and cell surface area. Co-treatment with sildenafil (1 μM, 48 h) inhibited these ET-1-induced hypertrophic responses. Although ET-1 enhanced the gene expression of TRPCs, sildenafil inhibited the enhanced gene expression of TRPC1, C3 and C6. Moreover, co-treatment with sildenafil abolished the augmentation of SOCE in the hypertrophied cardiomyocytes.
*Conclusions:* These results suggest that sildenafil inhibits cardiomyocyte hypertrophy by suppressing the up-regulation of TRPC expression.



## 1. Introduction

Cardiac hypertrophy is an adaptive response against an increased afterload that confers increased risk of cardiovascular-associated morbidity and mortality. A considerable number of signaling pathways has been proposed to induce cardiac hypertrophy, including an increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) [1–3]. Recent studies have established that transient receptor potential canonical channels (TRPCs) play an important role as a $Ca^{2+}$ entry pathway in calcineurin/nuclear factor of activated T cells (NFAT)-mediated cardiomyocyte hypertrophy [4–7]. Several reports showed that increased expression of TRPC3 and/or TRPC6 is involved in cardiac hypertrophy via receptor operated $Ca^{2+}$ entry (ROCE) [5,8,9]. NFAT binds to the TRPC6 promoter; thus, activation of TRPC6 enhances its own expression through a positive feedback mechanism [9]. Additionally, we showed that TRPC1 contributed to cardiomyocyte hypertrophy via store operated $Ca^{2+}$ entry (SOCE) and subsequent NFAT activation [4,7], in which the expression of TRPC1 was also increased. Conversely, $TRPC1^{-/-}$ mice failed to manifest evidence of maladaptive cardiac hypertrophy and maintained preserved cardiac function when subjected to hemodynamic stress and neurohormonal excess [6]. Therefore, up-regulation of TRPCs is currently recognized as a cause of cardiac hypertrophy.

The cGMP-specific phosphodiesterase type 5 (PDE5) inhibitor, sildenafil, blocks TRPC6 activation by phosphorylation at $Thr^{69}$ [10] and halts subsequent enhanced expression of TRPC6 [11], leading to the prevention of the cardiac hypertrophy [12–16]. Sildenafil is used clinically to treat pulmonary artery hypertension. A previous study revealed that sildenafil inhibits up-regulation of TRPC1 and leads to suppression of endothelin (ET)-1-mediated smooth muscle cell proliferation in pulmonary arteries [17]. These data raised the possibility that sildenafil regulates the expression of various TRPCs in cardiomyocytes. We tested the hypothesis that sildenafil blocks the up-regulation of TRPC1, 3, and 6 to suppress cardiomyocyte hypertrophy.

## 2. Materials and methods

### 2.1. Cell culture

The Animal Ethics Committee of the Akita University School of Medicine approved the study protocol. Cardiomyocytes from 1- to

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2-day-old Sprague–Dawley rats were isolated, subjected to Percoll gradient centrifugation, and cultured as described previously [18]. The purified cardiomyocytes were plated on 35 mm dishes ($1.6 \times 10^5$ cells per dish) in minimum essential medium (MEM) supplemented with 5% calf serum, penicillin (100 U/ml, GIBCO), and streptomycin (100 μg/ml, GIBCO). The cardiomyocyte cultures were incubated with ET-1, or ET-1 + sildenafil (0.1 μM, 10 μM, Pfizer) separately at 37 °C for 48 h in humidified air with 5% carbon dioxide, after which the medium was changed to serum-free MEM. The cell surface area was measured using NIH image (http://rsb.info.nih.gov/nih-image/).

### 2.2. Reverse transcriptase-polymerase chain reaction analysis

Total RNA was isolated from primary cultures and reverse transcription performed using standard methods. For reverse transcriptase-polymerase chain reaction (RT-PCR), 2.0 μl of template were used. Sequences specific for rat TRP (rTRP)-C1, -C3, -C5, -C6, BNP, ANF, and alpha skeletal actin (SKA) were amplified using the following primers: rTRPC1s (5′-TATGGGGAAGAACTGCAGTCC-3′), rTRPC1as (5′-CAGATCTTGGCGCAGTTCATT-3′), rTRPC3s (5′-TATGGTGGTCGTTCTGCTCA-3′), rTRPC3as (5′-CGATTTTGGACTAGGGACCA-3′), rTRPC4s (5′-AACCTAAGCAATGGTTCTGCC-3′), rTRPC4as (5′-CACCACCTTCTCCGACTTGAA-3′), rTRPC5s (5′-ATGGGTCCCTCTTTCAAGAAA-3′), rTRPC5as (5′-TTGTTCTTCCTGTCCATCACC-3′), rTRPC6s (5′-CTTGTGCCAAGTCCAAAGTCC-3′), rTRPC6as (5′-TTCCTTCAGCTCCCCTTCGTT-3′), rBNPs (5′-CAGAACAATCCACGATGCAG-3′) and rBNPas (5′-CGGTCTATCTTCTGCCCAAA-3′). As a control, β-actin cDNA was amplified over 25 cycles using rBactin1 (5′-AGGAAGGAAGGCTGGAAGAG-3′) and rBactin2 (5′-CAGCCTGGATGGCTACGTACA-3′) primers to produce a 211-bp fragment. Comparative RT-PCR reactions were amplified over 30 cycles, except for brain natriuretic peptide, which was done with 25 cycles.

### 2.3. Measurement of intracellular calcium concentrations

Calcium imaging was used to assay the formation of functional cation channels. Cardiomyocytes were incubated with the calcium indicator Fura-2AM (5 μM; Dojindo Laboratories, Kumamoto, Japan) at 37 °C for 60 min in HEPES-buffered saline (in mM: NaCl 136.9, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, D-glucose 11.1, and HEPES 5.0; pH 7.4). A calcium-free solution was made using the same HEPES buffered saline solution with EGTA (0.5 mM) substituted for the calcium. Changes in the $[Ca^{2+}]_i$ of individual cells were measured using an Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan) equipped with a Nikon epifluorescence microscope (TE2000-U; Nikon, Tokyo, Japan) and band-pass filters for wavelengths of 340 and 380 nm. Thapsigargin (1 μM)-stimulated SOC entry upon a change from $Ca^{2+}$-free conditions to 5 mM $Ca^{2+}$ was measured in the presence of 10 μM nifedipine without the acute effect of ET-1, as described previously [19,20].

### 2.4. NFAT promoter activity assay

An NFAT-green fluorescent protein (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 48 h incubation, the cells were visualized using confocal laser-scanning microscopy.

### 2.5. Statistics

Data are presented as means ± standard error of the mean (SE). Differences were evaluated using the unpaired Student's *t*-test, and $p < 0.01$ was considered to indicate statistical significance.

## 3. Results

### 3.1. Inhibition of the cardiomyocyte hypertrophic responses by sildenafil

We initially examined the anti-hypertrophic effect of sildenafil on NFAT activity and growth of cardiomyocytes. Primary cultured cardiomyocytes were treated with the pro-hypertrophic peptide ET-1 (10 nM) for 48 h, as described previously [1], in the presence of sildenafil at 0.1 and 1 μM. NFAT promoter activity was evaluated by the EGFP fluorescence intensity 48 h after transfection of a NFAT-EGFP reporter plasmid. Although ET-1 treatment led to increased NFAT-EGFP fluorescence (Fig. 1A), cardiomyocytes treated with ET-1 + sildenafil showed marginal NFAT activity, suggesting that sildenafil inhibited NFAT activation induced by ET-1 [10,11,17]. After 48 h of treatment with ET-1, cell surface area was analyzed as a marker of cardiomyocyte growth. As shown in Fig. 1B, the increase of cell surface area was significantly suppressed in cells treated with sildenafil for 48 h (0.1 or 1 μM) (cell surface area [Fold induction]: control 1.00 ± 0.04, ET-1 1.57 ± 0.12, sildenafil 0.1 μM 0.97 ± 0.07, ET-1 + sildenafil 0.1 μM

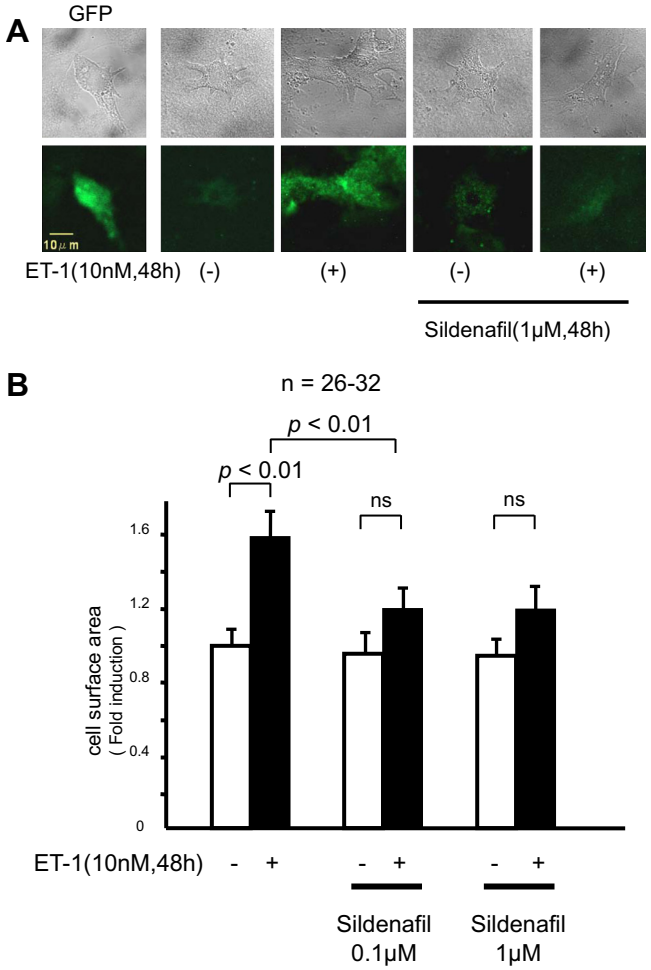


**Fig. 1.** (A) Representative EGFP fluorescence, indicating NFAT activation, and the corresponding differential interference contrast (DIC) images are shown. The NFAT promoter was activated by ET-1 (10 nM, 48 h), and sildenafil (1 μM, 48 h) inhibited ET-1-induced NFAT activation. (B) Cardiomyocyte surface area. Cardiomyocytes were treated with ET-1 (10 nM) and sildenafil (0.1 or 1 μM) as indicated in the lower panel. After 48 h, the surface area of ET-1 treated cells was significantly greater than that of control cells, suggesting inhibition by sildenafil. The results are presented as means ± SE of 26–32 cells. *$p < 0.01$.

1.19 ± 0.09, sildenafil 1 μM 0.99 ± 0.05, ET-1 + sildenafil 1 μM 1.13 ± 0.06). These findings suggest that both 0.1 and 1 μM sildenafil inhibited cardiomyocyte hypertrophy induced by ET-1.

### 3.2. Sildenafil inhibits the ET-1-induced up-regulation of TRPCs genes in cardiomyocytes

We examined the gene expression of TRPC1, 3, 4, 5 and 6 in ET-1 treated cardiomyocytes. As shown in Fig. 2, the expression levels of TRPC1, 3 and 6 were increased significantly by ET-1, although those of TRPC4 and TRPC5 were unchanged. Interestingly, when 0.1 or 1 μM sildenafil was added to cardiomyocytes, the increases in TRPC1, 3, and 6 expression were inhibited significantly, as expected. The change in TRPC expression levels was associated with a change in BNP, a known marker of cardiac hypertrophy. These results suggest that sildenafil inhibits the hypertrophic response and the expression of TRPC1, 3 and 6.

### 3.3. Inhibition of SOCE

Our previous studies revealed that SOCE is closely related to the expression of TRPC1, suggesting TRPC1-dependent SOCE. To confirm the inhibitory effects of sildenafil treatment for 48 h on TRPC channel expression, we analyzed SOCE in cardiomyocytes [4,21,22]. We examined SOCE in cardiomyocytes in the four groups (control, ET-1, sildenafil and ET-1 + sildenafil for 48 h). 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 nifedipine. Thapsigargin (1 μM)-stimulated SOCE upon a change from $Ca^{2+}$-free conditions to 5 mM $Ca^{2+}$ was measured. As shown in Fig. 3, there was a marginal change in SOCE between the group treated with only sildenafil and control. However, the increase in SOCE in the ET-1 group was inhibited significantly in cells treated with sildenafil (SOCE: control 0.33 ± 0.01, ET-1 0.59 ± 0.04, sildenafil 0.32 ± 0.01, ET-1 + sildenafil 0.36 ± 0.01). This result suggests that sildenafil inhibits SOCE during the development of cardiomyocyte hypertrophy.

Although we examined the acute effects of sildenafil on SOCE, SOCE in the ET-1 group was not inhibited by simultaneous sildenafil treatment (Supplemental data). These results suggest that sildenafil does not directly block SOC, but inhibits SOCE through suppressing TRPC1 expression in hypertrophied cardiomyocytes.

## 4. Discussion

In this study, sildenafil suppressed ET-1-induced hypertrophic responses, including NFAT activation, BNP expression and cell growth. Although sildenafil has been reported to inhibit TRPC6 activation and its expression [11], our results provide further evidence that sildenafil decreases TRPC1 and TRPC3 expression levels and SOCE in ET-1 treated cardiomyocytes. Therefore, it is likely that sildenafil prevents cardiomyocyte hypertrophy associated with suppression of TRPC expression and the subsequent decrease in $Ca^{2+}$ entry. Therefore, sildenafil could be a therapeutic option for cardiac hypertrophy, as well as pulmonary hypertension.

Sildenafil directly inhibits TRPC6 channel activation via protein kinase G-dependent phosphorylation, which results in a halt of the positive feedback mechanism that drives expression of TRPC6 [10]. These results suggest that sildenafil inhibits $Ca^{2+}$ entry through a genomic pathway, as well as non-genomic pathway. In this study,

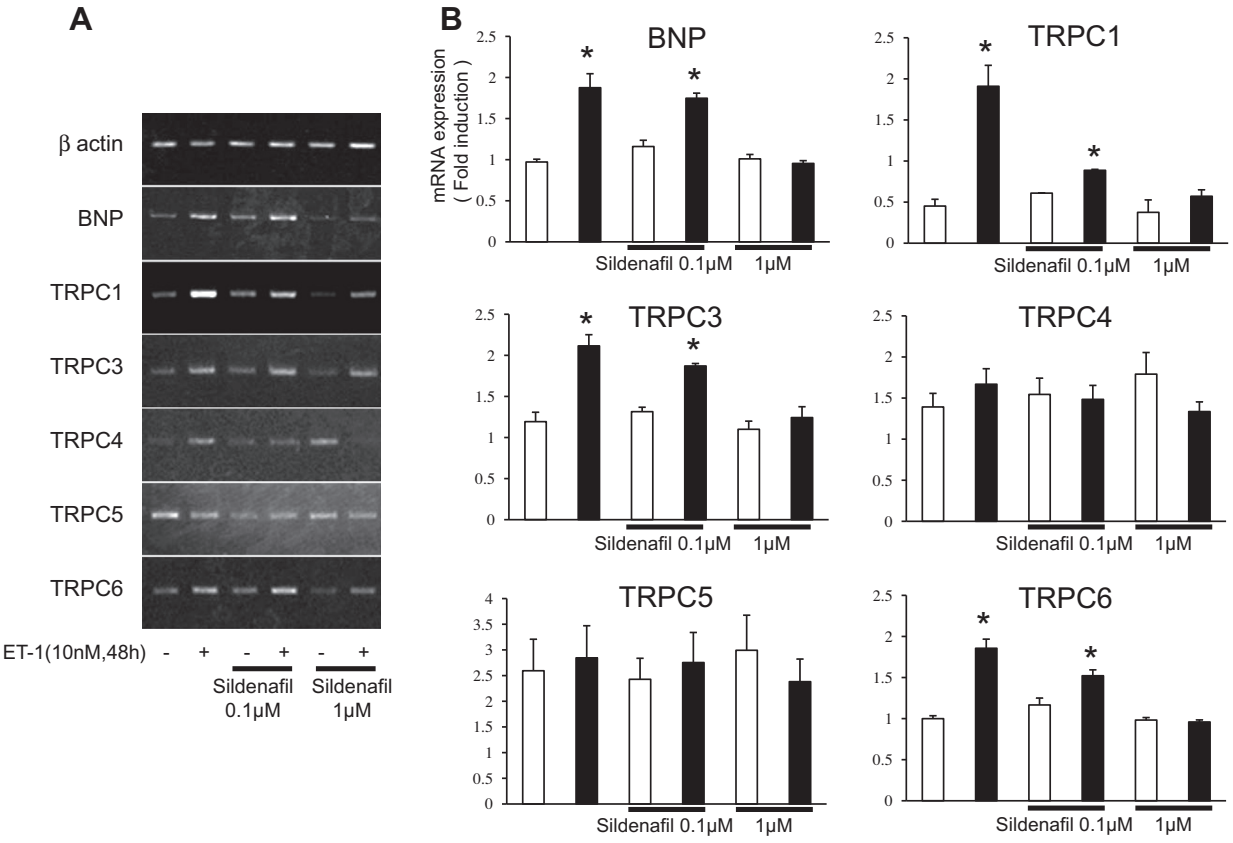


**Fig. 2.** Comparative RT-PCR analysis of β-actin, BNP, TRPC1, TRPC3, TRPC4, TRPC5 and TRPC6 expression. (A) Cardiomyocytes were treated with ET-1 (10 nM) and sildenafil (0.1 μM, 1 μM). After 48 h, RNA extraction and RT-PCR were performed. The results are representative of three independent experiments. (B) The statistical data for densitometry are presented as means ± SE. $^*p < 0.05$ vs. the control group.

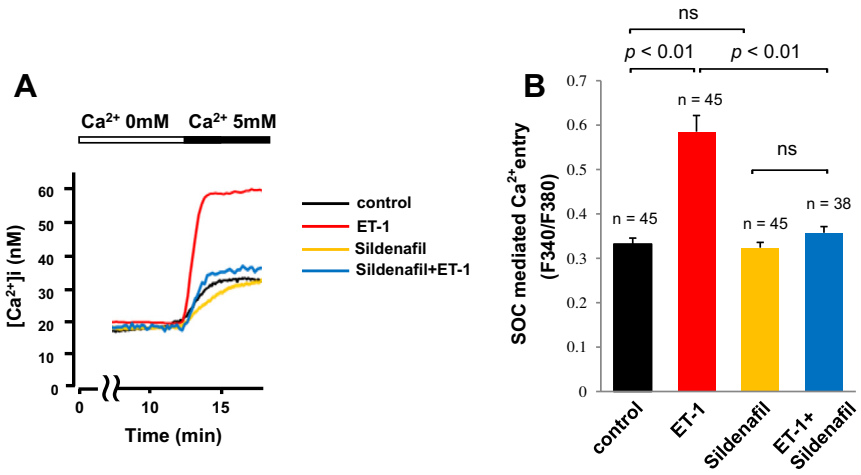


**Fig. 3.** (A) Representative recordings of SOCE in cardiomyocytes. (B) The statistical data are presented as means ± SE.

however, acute application of sildenafil did not block SOCE (Supplemental data). The inhibitory effect of sildenafil on SOCE was observed only following long-term treatment with sildenafil for 48 h, suggesting sildenafil inhibits SOCE by means of a genomic pathway. How does sildenafil suppress the expression of TRPCs? One possible explanation is that the blockade of TRPC6 by sildenafil halts the positive feedback mechanism for enhanced expression of TRPC1, -C3 and -C6. Because TRPC1, TRPC3, and TRPC6 have conserved NFAT consensus sequences in their promoters [4,9,23], once activated, TRPC-mediated $Ca^{2+}$ entry presumably activates NFAT and facilitates expression of other TRPCs. We believe that sildenafil acts as not only a direct TRPC6 blocker but also an inhibitor of the up-regulation of TRPCs during the development of cardiac hypertrophy.

Our results also suggested that sildenafil at concentrations as low as 0.1 μM was also effective in preventing cardiomyocyte hypertrophy. There has been controversy as to whether sildenafil inhibits only PDE5 or also suppresses PDE1 in cardiomyocytes [24]. Thus, while 0.1 μM sildenafil suppresses only PDE5, 1 μM sildenafil inhibits both PDE1 and PDE5. Our data suggest that sildenafil inhibits predominantly PDE5 rather than PDE1 in the hypertrophic response, which is consistent with previous reports [25].

In conclusion, sildenafil inhibits ET-1 induced cardiac hypertrophy by suppressing the up-regulation of TRPC expression. We believe that sildenafil is a potential therapeutic option for cardiac hypertrophy.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.002.

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