Biochemical and Biophysical Research Communications 436 (2013) 514–518

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Hironori Kiso [a], Takayoshi Ohba [b], Kenji Iino [a], Kazuhiro Sato [a], Yutaka Terata [a], Manabu Murakami [c], Kyoichi Ono [b], Hiroyuki Watanabe [a,*], Hiroshi Ito [a]

[a] Department of Internal Medicine, Division of Cardiovascular and Respiratory Medicine, Akita University Graduate School of Medicine, Japan
[b] Department of Cell Physiology, Akita University Graduate School of Medicine, Japan
[c] Department of Pharmacology, Hirosaki University Graduate School of Medicine, Japan

ARTICLE INFO

Article history:
Received 30 May 2013
Available online 10 June 2013

Keywords:
Sildenafil
Ca^{2+} entry
TRPC channels
Cardiomyocyte hypertrophy

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

* Corresponding author. Address: Department of Internal Medicine, Division of Cardiovascular and Respiratory Medicine, Akita University, Graduate School of Medicine, 1-1-1, Hondoh, Akita 010-8543, Japan.
E-mail address: hirow@doc.med.akita-u.ac.jp (H. Watanabe).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.06.002

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 (1.6×10^5 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

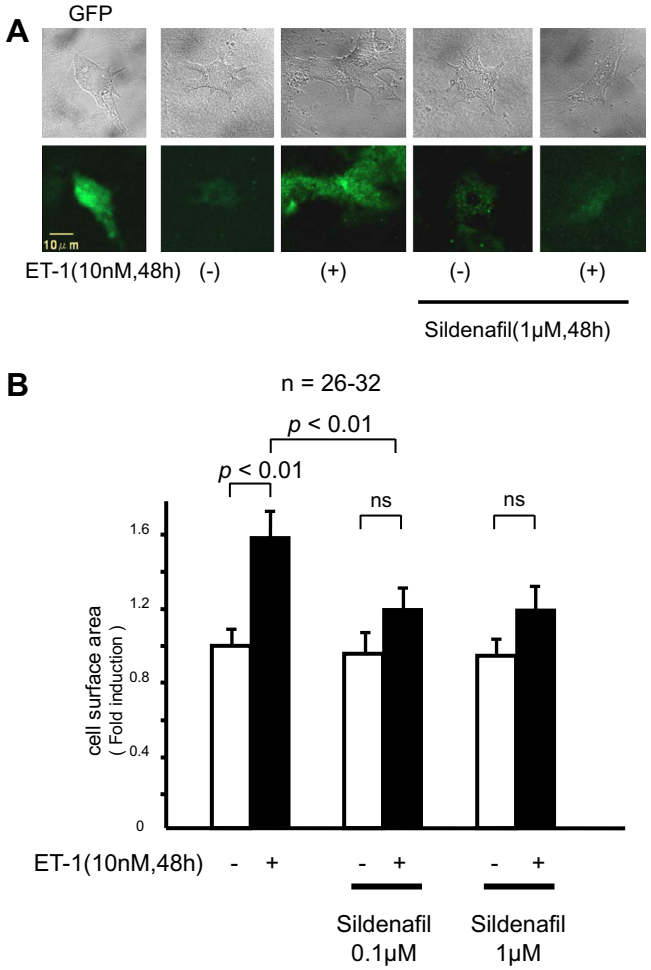

Fig. 1. (A) Representative EGFP fluorescence, indicating NFAT activation, and the corresponding differential interference contrast (DIC) images are shown. The NFAT promoter was activated by ET-1 (10 nM, 48 h), and sildenafil (1 μM, 48 h) inhibited ET-1-induced NFAT activation. (B) Cardiomyocyte surface 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. *$p < 0.01$.