Doctoral thesis (abridged version)

The role of TRPC6 in cardiac positive inotropy through sympathetic nervous system

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Introduction

Regulation of cardiac functions, primarily left ventricular (LV) contractility (inotropy) and heart rate (chronotropy), through autonomic nervous system is indispensable for maintenance of blood circulatory homeostasis. β adrenergic receptors (βARs) are expressed in the heart and activated by norepinephrine (NE) released from sympathetic nerve endings, which lead to induction of positive inotropic and chronotropic effects. Although β_1AR is believed to predominantly mediate NE-induced increase in cardiac functions, it is not well understood how cardiac functions are sufficiently fine-tuned by βARs, much lower-affinity binding receptors to NE than αARs.

Transient receptor potential canonical (TRPC) 6 is a component of receptor-operated and non-selective cation channel. Previous reports have shown that TRPC6 plays important roles in α_1AR-stimulated vasoconstriction and pathogenesis of heart failure in rodents. Although pathological roles of TRPC6 have been extensively studied, its physiological roles are still obscure. In this research, I investigated whether TRPC6 participates in physiological cardiac responses induced by activation of autonomic nervous system.

Materials and Methods

Animal experiments

All protocols using mice and rats were reviewed and approved by the ethics committees at the National Institutes of Natural Sciences and carried out in accordance with their guidelines. All mice were maintained in specific-pathogen-free area under a 12-h light/dark cycle.

For induction of hyperglycemia, male mice were injected intraperitoneally with streptozotocin (STZ, 50 mg/kg body weight) for 5 consecutive days. In isoproterenol (ISO) chronic injection experiments, ISO (30 mg/kg) filled in an osmotic mini pump was subcutaneously infused in male mice.

Hemodynamic parameters were measured using a micronanometer catheter (Millar Instruments).

For analyzing cardiovascular responses to receptor stimulation in autonomic nervous system, ISO (10 pg/g/min), acetylcholine (Ach, 0.5 mg/kg), NE (0.05 mg/kg) and hydralazine (0.5 mg/kg) were injected to mice from jugular vein.

Echocardiogrophy was performed using Nemio XG echocardiography (Toshiba) with a 14-MHz transducer.

Isolation of cardiomyocytes

For isolation of adult ventricular cardiomyocytes, heart was excised from heparinized and anesthetized mouse and the aorta was clamped. The clamped heart was perfused with Ca^{2+}-free isolation buffer and followed by enzyme mixture containing collagenase type 2 (1 mg/mL), trypsin (0.06 mg/mL), protease (0.06 mg/mL) with 0.3 mM $CaCl_2$. Thereafter, the LV chamber was cut into small pieces in enzyme mixture with 0.2 % bovine serum albumin and 0.7 mM $CaCl_2$. After cell-tissue suspension was filtered and centrifuged, the cell pellet was resuspended in isolation buffer containing 0.2% BSA and 1.2 mM $CaCl_2$ and incubated for 10 min at 37 °C. After final centrifugation, the cells were resuspended in tyrode solution.

Neonatal rat cardiomyocytes (NRCMs) were isolated from Sprague-Dawley rat pups on postnatal day 1-2. Dissected ventricles from pups were predigested in 0.05% trypsin-EDTA overnight and then digested in collagenase type 2 (1 mg/mL). After the dissociated cells were filtered and centrifuged, cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FBS and incubated for 90 min at 37 °C in a humidified atmosphere (5% CO_2, 95% air). After incubation, floating NRCMs were collected.

Isolated adult ventricular cardiomyocytes and NRCMs were plated to matrigel-coated culture dishes or glass base dishes.

Zinc and calcium imaging experiments

For analyzing changes in intracellular zinc ion (Zn^{2+}) concentration, cardiomyocytes and HEK293

cells were loaded with FluoZin-3 (2 μM), fluorescent Zn^{2+} indicator, for 30 min. For calcium transient measurement, cardiomyocytes were loaded with Fluo 4-AM (2 μM) for 30 min. Cells were field-stimulated at 1 Hz pacing frequency with a pulse of 10 msec and treated with ISO (10 nM).

cAMP measurements

For FRET imaging, NRCMs were transfected with Epac-based FRET sensor (gifted by Prof. K. Jalink, The Netherlands Cancer Institute), which detected changes in intracellular cyclic adenosine monophosphate (cAMP) concentration. Cells were stimulated with ISO (1 μM).

For ELISA experiment, NRCMs were treated with prazosin (10 μM), propranolol (10 μM) and methoxamine (1 μM) for 24 h with extracellular $ZnCl_2$ (50 μM). Thereafter, cells were treated with 3-isobutyl-1-methylxanthine (IBMX; 500 μM) for 1 h in the presence of each compound, following NE (1 μM) or ISO (1 μM) stimulation for 30 min. In N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) group, TPEN (25 μM) was added with IBMX. Intracellular cAMP levels were measured using cAMP EIA Kit (Cayman Chemical) according to manufacturer's instruction.

Proximity ligation assay

Proximity ligation assay (PLA) was conducted using Duolink PLA Fluorescence (Sigma) according to the manufacturer's instruction. After fixing and blocking, heart sections and cardiomyocytes were incubated with primary antibodies for 2 overnights at 4°C, followed by 1 h PLA probes incubation. The ligation (30 min) and amplification (150 min) steps were performed at 37 °C chamber.

Results

<Chapter 1> TRPC6 positively regulates cardiac inotropy by enhancing β adrenergic receptor signaling

First, cardiac and vascular responses induced stimulation of adrenergic receptors (βAR and αAR) or muscarinic receptor (MR) were compared among wild type (WT), TRPC3- and TRPC6-deficient

mice using surgical LV catheterization. I found that ISO (βAR specific agonist)-induced positive inotropic effect was reduced in TRPC6-deficient mice, whereas βAR-stimulated positive chronotropic effect, αAR-stimulated transient increase in blood pressure and MR-stimulated negative inotropic and chronotropic effects were not different significantly among three group. PLA revealed that TRPC6 was in close proximity to β_1AR in ventricle cardiomyocytes, but not sinoatrial node cells. Additionally, ISO-induced increase in shortening of sarcomere length and calcium transient was significantly reduced in isolated TRPC6-deficient adult mouse cardiomyocytes. Knockdown of TRPC6 suppressed ISO-mediated increase in intracellular cAMP production in NRCMs. These results suggest that TRPC6 acts as a positive regulator of βAR-Gs signaling in cardiomyocytes.

I next investigated the molecular mechanism underlying enhancement of βAR-Gs signaling by TRPC6 channel. I found that: 1) intracellular zinc ion (Zn^{2+}) pool in TRPC6-deficient cardiomyocytes was smaller than that in WT cardiomyocytes, 2) NE-induced αAR stimulation caused TRPC6-mediated Zn^{2+} influx in HEK293 cells. 3) αAR inhibition and intracellular Zn^{2+} chelation suppressed NE-induced cAMP production in NRCMs. 4) TRPC6 activation mediated by αAR stimulation enhanced ISO-induced cAMP production and this enhancement was canceled by TRPC6 knockdown in NRCMs. These results suggest that αAR-mediated Zn^{2+} influx via TRPC6 enhanced βAR-Gs signaling.

Finally, I examined whether TRPC6 contributes to baroreflex-induced cardiac inotropy in mice. I found that a reflex inotropy induced by hydralazine, a pharmacological vasodilator, was significantly reduced in TRPC6-deficient mice, while a coincident reflex chronotropy was not affected. I have previously reported that hyperglycemia increases TRPC6 expression in the heart. Consistently, positive inotropic effect was promoted in STZ-treated hyperglycemic mice. The interaction between TRPC6 and β_1AR determined by PLA was also enhanced in the base of hyperglycemic mouse heart. In Chapter 1, I revealed that TRPC6 positively regulates cardiac inotropy by enhancing βAR-Gs

signaling through αAR-Zn^{2+} axis.

<Chapter 2> TRPC6 is involved in ligand-stimulated β_1AR internalization process

Once βAR is activated by ligand binding and subsequent cell responses are triggered, receptor desensitization and internalization process is induced in order to keep a balance between receptor activation and signaling amplitude. In Chapter 2, I investigated an involvement of TRPC6 in βAR internalization process.

Co-expression of TRPC6 with β_1AR suppressed ISO-induced β_1AR internalization and β-arrestin 2 (βArr2) translocation to the plasma membrane in HEK293 cells. Basal interaction between β_1AR and βArr2 was significantly reduced in TRPC6-deficient mouse cardiomyocytes, indicating that TRPC6 is involved in ligand-stimulated βAR internalization process.

<Chapter 3> Involvement of TRPC6 in isoproterenol-induced heart failure

In Chapter 1 and 2, I have assessed acute βAR responses in several seconds to tens of minutes after stimulation. On the other hand, a chronic enhancement of βAR signaling is highly involved in pathogenesis of heart failure. I therefore investigated whether TRPC6 is involved in the progression of cardiac remodeling caused by chronic βAR activation.

Subcutaneous administration with ISO continuously for 4 weeks induced LV remodeling and dysfunction in WT mice, while ISO-induced heart failure was suppressed in TRPC6-deficient mice. In contrast, ISO-induced increase in heart weight was enhanced in TRPC6-deficient mice. In fact, chronic βAR activation enhanced TRPC6 mRNA expression in mouse hearts. Thus, TRPC6 contributes to heart failure and cardiac hypertrophy induced by chronic βAR activation in mice.

Discussion

Collectively, I revealed that TRPC6 links between αAR and βAR through Zn^{2+} mobilization, leading to enhancement of cardiac positive inotropy in response to sympathetic nerve activation. Since TRPC6

is expressed universally and various biological responses are regulated by sympathetic nervous system, Zn^{2+}-dependent enhancement of βAR signaling via TRPC6 might be involved in other physiological responses.