Journal of Molecular and Cellular Cardiology 56 (2013) 63–71

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

Decoy peptides targeted to protein phosphatase 1 inhibit dephosphorylation of phospholamban in cardiomyocytes

Jae Gyun Oh [a], Jihwa Kim [a], Seung Pil Jang [a], Mai Nguen [a], Dong Kwon Yang [a], Dongtak Jeong [b], Zee Yong Park [c], Sung-Gyoo Park [c], Roger J. Hajjar [b], Woo Jin Park [a,*]

[a] Global Research Laboratory, Gwangju Institute of Science and Technology, Gwangju, South Korea
[b] Cardiovascular Institute, Mount Sinai School of Medicine of New York University, New York, NY, USA
[c] College of Life Sciences, Gwangju Institute of Science and Technology, Gwangju, South Korea

ARTICLE INFO

Article history:
Received 7 May 2012
Received in revised form 5 December 2012
Accepted 7 December 2012
Available online 19 December 2012

Keywords:
SERCA2a
Phospholamban
Protein phosphatase 1
Decoy
Peptide

ABSTRACT

Cardiac sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) plays a crucial role in Ca^{2+} handling in cardiomyocytes. Phospholamban (PLB) is an endogenous inhibitor of SERCA2a and its inhibitory activity is enhanced *via* dephosphorylation by protein phosphatase 1 (PP1). Therefore, the inhibition of PP1-mediated dephosphorylation of PLB might be an efficient strategy for the restoration of reduced SERCA2a activity in failing hearts. We sought to develop decoy peptides that would mimic phosphorylated PLB and thus competitively inhibit the PP1-mediated dephosphorylation of endogenous PLB. The phosphorylation sites Ser16 and Thr17 are located within the flexible loop region (amino acids 14–22) of PLB. We therefore synthesized a 9-mer peptide derived from this region (ΨPLB-wt) and two pseudo-phosphorylated peptides where Ser16 was replaced with Glu (ΨPLB-SE) or Thr17 was replaced with Glu (ΨPLB-TE). These peptides were coupled to the cell-permeable peptide TAT to facilitate cellular uptake. Treatment of adult rat cardiomyocytes with ΨPLB-SE or ΨPLB-TE, but not with ΨPLB-wt, significantly elevated the phosphorylation levels of PLB at Ser16 and Thr17. This increased phosphorylation of PLB correlated with an increase in contractile parameters *in vitro*. Furthermore, the perfusion of isolated rat hearts with ΨPLB-SE or ΨPLB-TE, but not with ΨPLB-wt, significantly improved left ventricular developed pressure that had been previously impaired by ischemia. These data indicate that ΨPLB-SE and ΨPLB-TE efficiently prevented dephosphorylation of PLB by serving as decoys for PP1. Therefore, these peptides may provide an effective modality to regulate SERCA2a activity in failing hearts.

1. Introduction

Heart failure remains a leading cause of mortality and morbidity worldwide [1–3]. It is characterized by an increased ventricular chamber size and reduced systolic function of the heart. Previous studies support the notion that abnormalities in cardiac contractility cause the initiation and progression of heart failure [4–6]. The contractility of cardiomyocytes is directly regulated by intracellular Ca^{2+} cycling [7,8]. A small amount of extracellular Ca^{2+} enters cardiomyocytes through the voltage-dependent L-type Ca^{2+} channel and is then followed by a large release of Ca^{2+} from the sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR) [9]. This increase in intracellular Ca^{2+} triggers contraction of the myofilaments. Re-uptake of Ca^{2+} back into the SR through the SR Ca^{2+}-ATPase (SERCA) 2a and the Na^{+}/Ca^{2+} exchanger at the sarcolemma then initiates relaxation of the myofilaments.

Previous studies showed that decreased SERCA2a expression and activity are associated with heart failure in human and animal models [10–12]. Therefore, the restoration of SERCA2a levels by increasing the gene dosage was thought to be a rational approach for the treatment of heart failure. This proved to be the case when using heart failure models of rats [13–15] and pigs [16]. Furthermore, adeno-associated virus-mediated delivery of SERCA2a was recently shown to be a safe and effective modality for improving the cardiac functions in heart failure patients [17,18].

The activity of SERCA2a is negatively regulated by an endogenous inhibitor, phospholamban (PLB), which in turn is regulated by protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and protein phosphatase 1 (PP1). Phosphorylation at Ser16 and Thr17 of PLB by PKA and CaMKII, respectively, causes the disassociation of PLB from SERCA2a, permitting near-maximal Ca^{2+}-ATPase activity of SERC2a [19–21]. On the contrary, dephosphorylation of PLB at Ser16 or Thr17 by PP1 enhances the association between PLB and SERCA2a and the inhibition of SERCA2a by PLB [22,23]. Intriguingly, reduced PLB phosphorylation [24–26] concomitantly with an increased PP1 activity [27,28] have been observed in animal models and end-stage human heart failure. Therefore, normalization of PP1 activity and PLB

* Corresponding author at: College of Life Sciences, Gwangju Institute of Science and Technology (GIST), 1 Oryong-dong, Buk-gu, Gwangju, 500-712, South Korea. Tel.: +82 62 715 2564; fax: +82 62 715 2484.
E-mail address: ohjaegyun@gmail.com (W.J. Park).

0022-2828/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.yjmcc.2012.12.005

phosphorylation would be a reasonable approach to enhance cardiac function and suppress the progression of heart failure.

In this study, we synthesized small peptides that mimic phosphorylated PLB and thus competitively inhibit PP1-mediated dephosphorylation of endogenous PLB. Molecular decoys such as small peptides that mimic target proteins have been successfully utilized to interfere with protein–protein interactions [29], phosphorylation of target proteins by protein kinases [30], and dephosphorylation of phosphorylated proteins by phosphatases [31,32]. Our decoy peptides significantly elevated phosphorylation levels of PLB and increased contractile parameters *in vitro*. In addition, the decoy peptides significantly improved left ventricular developed pressure *ex vivo*, suggesting that these peptides provide an alternative modality for the restoration of SERCA2a activity in failing hearts.

2. Materials and methods

2.1. Decoy peptides

The decoy peptides were derived from the PLB protein sequence surrounding the phosphorylation sites Ser16 and Thr17: RAS^{16}T^{17}IEMPQ. The peptides were conjugated *via* a cystein–cystein bond at their N termini to the cell penetrating peptide TAT (YGRKKRRQRRR) to facilitate uptake into the cells. The peptides used in this study were as follows: ΨPLB-wt, RASTIEMPQ; ΨPLB-SE, RAETIEMPQ; ΨPLB-TE, RASEIEMPQ; ΨPLB-SD, RADTIEMPQ; ΨPLB-SETE, RAEEIEMPQ. In addition, shortened ΨPLB-SE peptides were also tested: 8-mer, AETIEMPQ; 7-mer, RAETIEM; 6-mer, RAETIE; 5-mer, RAETI. All of the peptides were synthesized and modified by AnyGen (Gwangju, Korea) and dissolved in distilled water to a stock concentration of 1 mM. Peptides were >95% pure. Adult cardiomyocytes were treated with 1 μM of decoy peptides for 1 h. Cell viability and morphology were not significantly affected by the peptides.

2.2. Isolation of adult rat ventricular myocytes

Ventricular myocytes were isolated from Sprague Dawley (SD) rat hearts as previously described [33] with minor modifications. Male rats of 8–12 weeks of age (250–320 g) were used. In brief, rats were anesthetized by the inhalation of 0.5% isofluran for 5 min. The heart was quickly removed from the chest and the aorta was retrogradely perfused at 37 °C for 3 min with calcium-free Tyrode buffer (137 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES [pH 7.4], 10 mM 2, 3-butanedione monoxime, and 5 mM taurine) gassed with 100% O_2. The enzymatic digestion was then initiated by adding 0.35 U/ml of collagenase type B (Roche) and 0.1 mg/ml of hyaluronidase (Worthington) to the perfusion solution. When the heart became swollen after 10 min of digestion, the left ventricle was quickly removed, cut into several chunks, and further digested in a shaker (60–70 rpm) for 10 min at 37 °C in the same enzyme solution. The supernatant containing the dispersed myocytes was then filtered through a cell strainer (100 μm in pore size, BD Falcon) and gently centrifuged at 500 rpm for 1 min. Extracellular Ca^{2+} was incrementally added back to a concentration of 1.25 mM over a span of 30 min to avoid the Ca^{2+} paradox. This procedure usually yielded ≥80% viable rod-shaped ventricular myocytes with clear sarcomere striations. Myocytes with obvious sarcolemmal blebs or spontaneous contractions were discarded.

2.3. Fluorescence microscopy

To confirm cellular uptake of the peptides, ΨPLB-SE was labeled with FITC. The isolated cardiomyocytes were plated onto a laminin-coated glass plate and cultured in modified Eagle's Medium (MEM) with Hanks' Balanced Salt solution, supplemented with 2 mM of L-carnitine, 5 mM of creatine and 5 mM of taurine, and 100 IU/ml of penicillin. The cells were exposed to 1 μM of FITC-labeled ΨPLB-SE for 1 h, and then washed twice with Tyrode solution. Fluorescence images were visualized using a Leica DMRBE microscope equipped with a 63× (1.4NA) oil objective and fluorescein FITC-optimized filter sets (OmegaR Optical Inc.). Images were acquired using a CoolSNAP TMfx CCD camera and analyzed with Metamorph imaging software (Universal Imaging Co.).

2.4. Western blot analysis

Heart lysates (50 μg) in SDS sample buffer were run on a SDS-PAGE gel and then transferred to a PVDF membrane (Bio-Rad). The membrane was blocked with a 5% of skim milk solution and then incubated overnight with antibodies directed against phospholamban (PLB) (Affinity Bioreagents), phospho-PLB (Ser16, Cell Signaling), phospho-PLB (Thr17, Badrilla), SERCA2a (Santa Cruz), Caspase 3 (cell signaling), or GAPDH (Santa Cruz). The membranes were then incubated with a secondary antibody conjugated to horseradish peroxidase (Jackson Immuno Research) and developed using the Western Lighting chemiluminescence reagent (Perkin Elmer).

2.5. Neonatal cardiomyocyte culture and in vitro gene transfer

Primary cardiomyocyte cultures were prepared from 1-day-old SD rats. Briefly, ventricular tissue was enzymatically dissociated, and the resulting cell suspension was enriched for cardiomyocytes using Percoll (Amersham Pharmacia) gradient centrifugation. Isolated cardiomyocytes were plated onto collagen-coated culture dishes (Corning) and cultured in DMEM medium (supplemented with 10% of FBS, 2 mM of L-glutamine, and 100 μM of bromodeoxyuridine (GIBCO BRL)). The medium of cardiac myocytes was changed to DMEM 12 h before gene transfer. Then, the mixture of pcDNA-PP1β (1 μg of plasmid DNA per plate) and LipofectAMINE (Invitrogen, US) was added into the plates. After incubation at 37 °C in 5% CO_2 for 4 h, the transfer medium was removed and replaced with DMEM medium with supplement. The transferred cells were cultured further for 48 h.

2.6. Cell contractility and intracellular Ca^{2+} transient measurements

The mechanical properties of ventricular myocytes were assessed using a video-based edge detection system (IonOptix), as previously described [34]. In brief, laminin-coated coverslips with attached cells were placed in a chamber mounted on the stage of an inverted microscope (Nikon Eclipse TE-100F) and perfused (about 1 ml/min at 37 °C) with Tyrode buffer (137 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES [pH 7.4]). For better observation of excitation–contraction coupling regulation, the cells were field-stimulated at a frequency of 3 Hz (30 V) using a STIM-AT stimulator/thermostat placed on a HLD-CS culture chamber/stim holder (Cell Micro Controls). The myocyte of interest was displayed on a computer monitor using an IonOptix MyoCam camera, which rapidly scanned the image area every 8.3 ms, so that the amplitude and velocity of shortening or relengthening were recorded with fidelity. Changes in cell length during shortening and relengthening were captured and analyzed using soft edge software (IonOptix).

To determine changes in Ca^{2+} transient, the cardiomyocytes were loaded with 0.5 μM of Fura2-AM (Molecular Probes), a Ca^{2+}-sensitive indicator, for 15 min at 37 °C. Fluorescence emissions were recorded simultaneously with the contractility measurements using IonOptix. Cardiomyocytes were exposed to light emitted by a 75 W halogen lamp through either a 340 or 380 nm filter while being field-stimulated as described above. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after initial illumination at 340 nm for 0.5 s and then at 380 nm for the duration of the recording protocol. The 340 nm excitation scan was then repeated at the end of the protocol, and qualitative changes in the intracellular Ca^{2+}