Journal of Molecular and Cellular Cardiology 112 (2017) 104–113

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Endothelial specific SIRT3 deletion impairs glycolysis and angiogenesis and causes diastolic dysfunction

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ARTICLE INFO

Keywords:
SIRT3
PFKFB3
Metabolic reprogram
Coronary microvascular dysfunction
Diastolic dysfunction

ABSTRACT

Endothelial glycolysis plays a critical role in the regulation of angiogenesis. We investigated the role of Sirtuin 3 (SIRT3) on endothelial cell (EC) glycolytic metabolism, angiogenesis, and diastolic function. Our aim was to test the hypothesis that loss of SIRT3 in ECs impairs endothelial glycolytic metabolism and angiogenesis and contributes to myocardial capillary rarefaction and the development of diastolic dysfunction. Using SIRT3 deficient ECs, SIRT3 was found to regulate a metabolic switch between mitochondrial respiration and glycolysis. SIRT3 knockout (KO)-ECs exhibited higher mitochondrial respiration and reactive oxygen species (ROS) formation. SIRT3 knockout (KO)-ECs exhibited a reduction in the expression of glycolytic enzyme, PFKFB3, and a fall in glycolysis and angiogenesis. Blockade of PFKFB3 reduced glycolysis and downregulated expression of VEGF and Angiopoietin-1 (Ang-1) in ECs. Deletion of SIRT3 in ECs also impaired hypoxia-induced expression of HIF-2α, VEGF, and Ang-1, as well as reduced angiogenesis. In vivo, endothelial-specific SIRT3 KO (ECKO) mice exhibited a myocardial capillary rarefaction together with a reduced coronary flow reserve (CFR) and diastolic dysfunction. Histologic study further demonstrated that knockout of SIRT3 in ECs significantly increased perivascular fibrosis in the coronary artery. These results implicate a role of SIRT3 in modulating endothelial function and cardiac function. Ablation of SIRT3 leads to impairment of EC glycolytic metabolism and angiogenic signaling, which may contribute to coronary microvascular rarefaction and diastolic dysfunction in SIRT3 ECKO mice.

Subject codes

Coronary circulation
Epigenetics
Echocardiography
Heart failure
Metabolism

1. Introduction

Heart failure (HF) is a high prevalent, progressive disease that develops with advanced age, hypertension, and diabetes [1–4]. Heart failure is typically divided into two phenotypes, heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF). More than half of newly diagnosed heart failure patients present with preserved ejection fraction [5,6]. Standard of care medications for HFrEF have long been established, but thus far, large clinical trials have failed to show any significant benefit in life expectancy in patients with HFpEF [7]. Despite the clinical importance of HFpEF, our understanding of its pathophysiology and molecular mechanism is incomplete.

Endothelial (EC) dysfunction is highly prevalent in HFpEF patients and is correlated with exercise intolerance [8,9]. A recent clinic study indicates that microvascular rarefaction is a major contributor to diastolic dysfunction in HFpEF patients [3]. SIRT3 is predominately localized in the mitochondria of the heart, has emerged as a novel regulator

Abbreviations: 2-DG, 2-deoxyglucose; 3PO, 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one; Ang-1, Angiopoietin-1; CFR, Coronary flow reserve; DHE, Dihydroethidium; ECAR, Extracellular acidification rate; DMSO, Dimethyl sulfoxide; ECKO, Endothelial-specific SIRT3 knockout; ECM, Extracellular basement membrane matrix; ET, Ejection fraction; HIF, Hypoxia-inducible factor; HFpEF, Heart failure with preserved ejection fraction; HFrEF, Heart failure with reduced ejection fraction; IB4, Isolectin B4; IVRT, Isovolumic relaxation time; IVCT, Isovolumic contraction time; LCA, Left proximal coronary artery; LV, Left ventricle; MPI, Myocardial performance index; PFKFB3, 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase isoform 3; PW Doppler, Pulsed-wave Doppler; ROS, Reactive oxygen species; SIRT3, Sirtuin3; VEGF, Vascular endothelial growth factor; vWF, von Willebrand factor; WT, Wild-type

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http://dx.doi.org/10.1016/j.yjmcc.2017.09.007
Received 2 August 2017; Received in revised form 12 September 2017; Accepted 15 September 2017
Available online 19 September 2017
0022-2828/

of mitochondrial function and cellular metabolism [4,10,11]. SIRT3 levels were decreased as much as 40% in elderly adults with sedentary lifestyles compared to younger individuals [12]. Our recent study indicated that a fall in SIRT3 levels is associated with obesity-induced microvascular rarefaction and cardiac dysfunction [13]. Gene therapy with the angiogenic growth factor, apelin, increased myocardial vascular density and attenuated ischemia-induced cardiac dysfunction in diabetic STZ mice, but had no effect in STZ-SIRT3 knockout (KO) mice [14]. These studies indicate that loss of SIRT3 contributes to development of heart failure. Endothelial metabolism plays a critical role in the regulation of angiogenesis since ECs depend on glycolysis for migration and proliferation [15,16]. The 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3 (PFKFB3) is a key regulator of glycolysis in endothelial cells (EC), that has been shown to promote angiogenic sprouting [15,17,18]. Our recent study demonstrated a significant reduction of PFKFB3 expression and impaired angiogenesis in the heart of SIRT3 KO mice [19]. However, a direct role of SIRT3 in endothelial glycolytic metabolism, angiogenesis and diastolic function is unknown and has not been studied previously.

We hypothesized that endothelial SIRT3 regulates the glycolytic metabolism for maintaining coronary microvascular function and diastolic function. In the absence of SIRT3, the glycolytic metabolism of EC is reduced, which contributes to impairment of angiogenesis and coronary microvascular dysfunction and diastolic dysfunction. To test this hypothesis, we investigated the cardiac phenotype of endothelial-specific SIRT3 knockout (SIRT3 ECKO) mice and examined endothelial glycolytic metabolic profiles in SIRT3 KO-ECs.

2. Materials and methods

2.1. Chemicals and reagents

Dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (MO, USA). Geltrex® LDEV-free reduced growth factor basement membrane matrix (ECM) was from Invitrogen (Life Technologies, NY). L-glutamine (200 mM) and sodium pyruvate (100 mM) were from GIBCO (Invitrogen, Life Technologies, NY). 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) was from EMD Millipore (MA, USA). Dihydroethidium (DHE) was from Molecular Probes (OR, USA).

2.2. Animals

All animals were fed with laboratory standard chow and water, and housed in individually ventilated cages in the Laboratory Animal Facilities at the University of Mississippi Medical Center. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi Medical Center (Protocol ID: 1280B) and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

2.3. Generation of SIRT3 ECKO mice

SIRT3 ECKO mice were generated using the Cre-LoxP system as depicted in Fig. S1A. $SIRT3^{flox/flox}$ mice were originally obtained from Dr. Eric Verdin at Gladstone Institute of Virology and Immunology, University of California. The exons 2 and 3 of SIRT3 gene are flanked with LoxP sites, priming for subsequent deletion by Cre recombinase. $SIRT3^{flox/flox}$ mice were crossbred with VE-Cadherin-Cre (Cdh5-Cre) transgenic mice [B6.FVB-Tg(Cdh5-cre)7Mlia/J from Jackson Laboratories] expressing Cre recombinase in vascular endothelial cells (Fig. S1A). The resulting Cdh5-Cre/$SIRT3^{flox/-}$ heterozygous mutants were then mated with $SIRT3^{flox/flox}$ to obtain endothelial-specific ablated SIRT3 mutant mice (SIRT3 ECKO). Only male mice (12 month of age) were used for all the experiments and $SIRT3^{flox/flox}$ (SIRT3 LoxP) mice were used as the corresponding control. Genotyping was performed by tail DNA PCR analysis. Primer sequences used for genotyping floxed SIRT3 allele were as follows: SIRT3 Forward 5′-TACTGAATATCAGTGGGAACG-3′, SIRT3 Reverse 5′-TGCAACAAG GCTTTATCTTCC-3′, and SIRT3 WT Forward 5′-CTTCTG CGGCTCTATACACAG-3′. Cdh5-Cre transgene was detected using the following primers: transgene forward 5′-GCGGTCTGGCA GTAAAAACTATC-3′ and transgene reverse 5′-GTGAAACAGCAT TGCTGTCACTT-3′; internal positive control forward 5′-CTAGGCCAC AGAATTGAAAGATCT-3′ and internal positive control reverse 5′-GTAGGTGGAAATTCT AGCATCATCC-3′. PCR products were analyzed on 1.5% tris-acetate-EDTA (TAE) agarose gels (Fig. S1B and C). To confirm that SIRT3 was knocked out at protein level, the isolated endothelial cells from SIRT3 LoxP and SIRT3 ECKO mice were co-stained with rabbit anti-SIRT3 antibody (ab86671, Abcam, MA) and Alexa Fluor® 488 conjugated *Griffonia Bandeiraea Simplicifolia* Isolectin B4 (1:50; IB4, Invitrogen, OR), followed by incubation with anti-rabbit Cy™3 conjugated secondary antibody (Jackson ImmunoResearch Inc., PA). Pictures were taken using a Nikon Eclipse 80i microscope, coupled with an X-Cite® 120 Fluorescence Illumination system (Nikon Instruments, NY). The absence of SIRT3 was also confirmed by immunoblot analysis.

2.4. Echocardiography

Transthoracic echocardiograms were performed on SIRT3 LoxP and SIRT3 ECKO using a Vevo770 High-Resolution In Vivo Micro-Imaging System equipped with a RMV 710B scanhead (VisualSonics Inc., Canada). The studied mouse was anesthetized by inhalation of 1.5–2% isoflurane mixed with 100% medical oxygen administered with a vaporizer in an isolated chamber for induction. Anesthesia was maintained with 1–1.5% isoflurane with a heart rate of ~ 400–450 beats per min (bpm).

For LV diastolic function, transmitral inflow Doppler images are obtained in apical 4-chamber (A4C) view using pulsed-wave (PW) Doppler mode to measure the ratio of peak velocity of early to late filling of mitral inflow (E/A), isovolumic relaxation time (IVRT), isovolumic contraction time (IVCT) and ejection time (ET) [20]. The myocardial performance index (MPI), is calculated as the following formula: MPI = (IVRT + IVCT)/ET. In addition, Tissue Doppler images are obtained from the mitral annulus to measure tissue motion velocity in early and late diastole (E′ and A′, respectively).

For coronary flow reserve, the left proximal coronary artery (LCA) is visualized in a modified parasternal LV short-axis view, and cine loop of LCA is recorded at baseline, and under hyperemic conditions induced by inhalation of 1% and 2.5% isoflurane, respectively. The coronary flow reserve (CFR) is calculated as the ratio of hyperemic peak diastolic flow velocity to baseline peak diastolic flow velocity [19,21].

2.5. Primary EC culture and treatment

Microvascular endothelial cells (MECs) were isolated from WT or SIRT3 KO mice (n = 3) as described previously [22]. In brief, The WT and SIRT3 KO mice were anesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg). The lung was perfused with 10 ml chilled PBS containing 2.5 mM EDTA, followed by perfusion of 5 ml of chilled 0.25% trypsin/2 mM EDTA through the right ventricle of the heart. The heart and lung were then removed *en bloc* and incubated at 37 °C for 20 min. Small cuts were made in the visceral plura and the ECs can be harvested by pipetting 1.5 ml EGM-2 medium (Lonza, MD) supplemented with growth factors and 10% fetal bovine serum (FBS) up and down 10–15 times. The cell suspension was filtered through a 100 μm filter and plated into 60 mm cell culture dish. After 3 days of incubation, ECs formed small colonies and non-ECs were removed by vacuum until > 90% ECs left. Purity of these cells was checked by staining with rabbit anti-von Willebrand factor (vWF) polyclonal antibody (Santa Cruz biotechnology, TX) (Fig. S2A). The absence of the expression of