Journal of Molecular and Cellular Cardiology 107 (2017) 41–51

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Non-nuclear estrogen receptor alpha activation in endothelium reduces cardiac ischemia-reperfusion injury in mice

Sara Menazza [a,1,2], Junhui Sun [a,2], Swathi Appachi [a], Ken L. Chambliss [b], Sung Hoon Kim [c], Angel Aponte [d], Sohaib Khan [e], John A. Katzenellenbogen [c], Benita S. Katzenellenbogen [f], Philip W. Shaul [b], Elizabeth Murphy [a,*]

[a] Systems Biology Center, National Heart Lung and Blood Institute, NIH, Bethesda, MD, United States
[b] Department of Pediatrics, UT Southwestern Medical Center, Dallas, TX, United States
[c] Department of Molecular and Integrative Physiology, United States
[d] Proteomics Core, NHLBI, NIH, Bethesda, MD, United States
[e] Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL, United States
[f] University of Cincinnati Cancer Center, Cincinnati, OH, United States

ARTICLE INFO

Article history:
Received 7 March 2017
Received in revised form 24 April 2017
Accepted 25 April 2017
Available online 27 April 2017

Keywords:
Cardiomyocyte
Endothelium
Estrogen receptor
Nitric oxide signaling

ABSTRACT

Steroid hormone receptors including estrogen receptors (ER) classically function as ligand-regulated transcription factors. However, estrogens also elicit cellular effects through binding to extra-nuclear ER (ERα, ERβ, and G protein-coupled ER or GPER) that are coupled to kinases. How extra-nuclear ER actions impact cardiac ischemia-reperfusion (I/R) injury is unknown. We treated ovariectomized wild-type female mice with estradiol or an estrogen-dendrimer conjugate (EDC), which selectively activates extra-nuclear ER, or vehicle interventions for two weeks. I/R injury was then evaluated in isolated Langendorff perfused hearts. Two weeks of treatment with estradiol significantly decreased infarct size and improved post-ischemic contractile function. Similarly, EDC treatment significantly decreased infarct size and increased post-ischemic functional recovery compared to vehicle-treated hearts. EDC also caused an increase in myocardial protein S-nitrosylation, consistent with previous studies showing a role for this post-translational modification in cardioprotection. In further support of a role for S-nitrosylation, inhibition of nitric oxide synthase, but not soluble guanylyl cyclase blocked the EDC mediated protection. The administration of ICI182,780, which is an agonist of G-protein coupled estrogen receptor (GPER) and an antagonist of ERα and ERβ, did not result in protection; however, ICI182,780 significantly blocked EDC-mediated cardioprotection, indicating participation of ERα and/or ERβ. In studies determining the specific ER subtype and cellular target involved, EDC decreased infarct size and improved functional recovery in mice lacking ERα in cardiomyocytes. In contrast, protection was lost in mice deficient in endothelial cell ERα. Thus, extra-nuclear ERα activation in endothelium reduces cardiac I/R injury in mice, and this likely entails increased protein S-nitrosylation. Since EDC does not stimulate uterine growth, in the clinical setting EDC-like compounds may provide myocardial protection without undesired uterotrophic and cancer-promoting effects.

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1. Introduction

It is well established that pre-menopausal women have reduced incidence of cardiovascular disease (CVD) compared with men, but the incidence of CVD rises following menopause, suggesting a role for sex hormones in the reduction in CVD in pre-menopausal females [1,2]. However, a large clinical trial, the Women's Health Initiative (WHI) found that treating post-menopausal women with estrogen or estrogen plus progesterone was not beneficial [3,4]. A recent update of the WHI examined different age groups and concluded that although conjugated equine estrogens had harmful effects on older women, there were some beneficial effects on myocardial infarction incidence in younger menopausal women [5]. The reasons for the age-dependent effects are unclear [6,7], and at least a portion of the current dilemmas regarding

Abbreviations: CVD, Cardiovascular disease; EDC, Estradiol-dendrimer conjugate; eNOS, Endothelial nitric oxide synthase; ER, Estrogen receptor; GPER, G protein-coupled estrogen receptor; GPR30, Orphan G-protein coupled receptor; I/R, Ischemia/Reperfusion; LVDP, Left ventricular developed pressure; OVX, Ovariectomy; RPP, Rate pressure product; SERMs, selective estrogen receptor modulators; SNO, S-nitrosylation; TTC, 2,3,5-triphenyltetrazolium chloride; WHI, Women's Health Initiative.

* Corresponding author at: National Heart Lung and Blood Institute, NIH, Building 10, Rm 6N248B, 10 Center Drive, Bethesda, MD 20892, United States.
E-mail address: murphy1@mail.nih.gov (E. Murphy).

[1] Present address: Department of Biomedical Sciences, University of Padova, Padova, Italy.

[2] These authors contributed equally.

http://dx.doi.org/10.1016/j.yjmcc.2017.04.004
0022-2828/Published by Elsevier Ltd.

the clinical use of estrogen-based therapies resides in insufficient understanding of estrogen actions in the heart. Of note, the identity of the operative estrogen receptor(s) and the signaling mechanisms involved in estrogen-mediated protection against ischemia-reperfusion (I/R) injury are poorly understood. A better understanding of these processes could have important implications on the development of selective estrogen receptor modulators (SERMs) to provide beneficial effects of estrogen without unwanted uterotrophic and cancer-promoting effects [8,9].

Estrogen signaling is mediated by estrogen binding to estrogen receptors (ER). There are two classic nuclear ER (ERα and ERβ) and a G-protein coupled receptor known as GPR30 or G-protein estrogen receptor (GPER). ERα and ERβ classically bind estrogen and translocate to the nucleus where they modulate transcription. ERα and ERβ also have extra-nuclear actions involving the activation of kinase signaling, leading to acute cellular responses as well as alterations in gene expression that occur via kinase activation [10–15]. On binding estrogen, GPER, localized at the plasma membrane, also activates signaling cascades [16–19].

Although it is well-recognized that estrogens have favorable impact on the severity of cardiac I/R injury, the role of extra-nuclear actions of estrogens and estrogen receptors in this protection is unknown. The goal of the present study was to determine how extra-nuclear estrogen actions impact cardiac I/R injury. This was accomplished using an estrogen-dendrimer conjugate (EDC) comprised of a non-degradable poly(amido)amine dendrimer to which estradiol molecules are linked by a stable covalent bond [20]. Previous studies have shown that EDC is excluded from the nucleus and it stimulates extra-nuclear ER signaling but is ineffective in modulating nuclear ER transcriptional activity, and the selectivity has been demonstrated both in cell culture and in vivo in mice [21]. We found that EDC treatment was as protective as estradiol and it significantly decreased infarct size and increased post-ischemic functional recovery compared to vehicle-treated hearts. We further showed that endothelial ERα, but not cardiomyocyte ERα was required for the protection.

2. Material and methods

2.1. Animals

All animals were treated and cared for in accordance with the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH), Revised 2011], and protocols were approved by the Institutional Animal Care and Use Committee of the National Heart Lung and Blood Institute. Female C57BL/6J mice, obtained from Jackson Laboratories (Bar Harbor, ME), were bilaterally ovariectomized at 10 weeks of age and delivered to the laboratory at 11 weeks of age. Heterozygous C57BL/6 floxed ERα mice (*flox*|+) originally generated by S.A. Khan were kindly provided by L. Hahner and D. Clegg [22,23]. Selective deletion of ERα from cardiac myocytes was accomplished using tamoxifen-inducible α-myosin heavy chain *cre*-transgenic mice (Mer-Cre-Mer, Jackson Laboratories, Bar Harbor, ME). In another group of mice the receptor was silenced in endothelial cells using vascular endothelial cadherin promoter-driven Cre mice (VECad-Cre) [24].

To generate cardiomyocyte-specific ERα knockout (cs-ERαKO) mice, mice heterozygous for the Exon 3-floxed ERα allele (*flox*|+) [23] were crossed to obtained homozygous mice for the floxed ERα alleles (*flox*|*flox*). Heterozygous *flox*| + mice were also crossed with Mer-Cre-Mer mice, and cardiomyocyte-specific ERα knockouts were then generated by crossing *cre*|+,*flox*| + mice with *flox**flox* mice. The presence of the *cre* transgene and the modified *loxP* alleles were verified by PCR of digested genomic DNA. For the *cre* transgene, the following PCR primers were used: reverse, 5′-AGGTGGACCTGATCATGGAG-3′ and forward, 5′-ATACCGGAGATCATGCAAGC-3′. We also used an internal positive control, performing PCR with the following primers: 5′-CTAGGCCACAGAATTGAAAGATCT-3 (forward) and 5′- GTAGGTGGAAA TTCTAGCATCATCC-3 (reverse). The primers used for identifying the presence of the *loxP* site are as follows: 5′- TGGGTTGCCC GATAACAATAAC -3′ (forward) and 5-AAGAGATGTAGGGCGGGAAAAG-3′ (reverse). The expression of ERα in the hearts of Cre negative control cs-ERαWT mice and Cre positive cs-ERαKO mice was quantified by Western blotting using ERα specific antibody (sc-8005, Santa Cruz).

Mice with endothelial cell-specific deletion of ERα (es-ERαKO) were generated by crossing ERα floxed mice [23] with vascular endothelial cadherin promoter-driven Cre mice (VECad-Cre) [24]. In addition to genotyping done on tail-derived DNA, genotyping was performed on DNA isolated from intact versus endothelium-denuded aorta samples to evaluate effective ERα gene excision in the endothelium.

2.2. Minipump agent administration

Two weeks after bilateral ovariectomy (OVX), micro-osmotic Alzet pumps (model 1002, DURECT Corporation, Cupertino, California) were implanted subcutaneously into female mice (Fig. 1). Mice were anesthetized using 1–3% isoflurane given by inhalation through a vaporizer. Each pump delivered a constant dose (0.25 μl/h) of vehicle, estradiol, EDC, or dendrimer. Six μg of estradiol or estradiol equivalents (for EDC) were dispensed daily. ICI 182,780 was infused at 2 mg/kg/day [25]. Following two weeks of treatment, hearts were excised for the I/R protocol (Fig. 1A).

2.3. I/R protocol, post-ischemic functional recovery, and infarct size determination

Female mice were anesthetized with sodium pentobarbital (50 mg/kg) and anti-coagulated with heparin (1000 USP units/ml), injected directly into the inferior vena cava. Hearts were excised quickly and placed in ice-cold Krebs-Henseleit buffer (in mmol/l: 120 NaCl, 11 D-glucose, 25 $NaHCO_3$, 1.75 $CaCl_2$, 4.7 KCl, 1.2 $MgSO_4$, and 1.2 KH_2PO_4). The aorta of the heart was then cannulated to a Langendorff perfusion apparatus. Once cannulated, the heart was retrograde perfused with Krebs-Henseleit buffer at a temperature of 37 °C and a constant pressure of 100 cm of water. Krebs-Henseleit buffer was oxygenated with 95% O_2/5% CO_2 and maintained at pH 7.4. For all treatment groups, hearts were equilibrated for 20 min, subjected to 30 min of ischemia, and reperfused for 90 min. L-NAME (10 μM) and ODQ (10 μM) were added to the perfusate 10 min prior to ischemia and were present during ischemia and the first 30 min of reperfusion. To monitor functional recovery during the I/R protocol, a latex balloon connected to a pressure transducer was inserted into the left ventricle. Left ventricular developed pressure (LVDP) was recorded using a PowerLab system (ADInstruments, Colorado Springs, CO). Rate pressure product (RPP) was determined by multiplying LVDP by heart rate. Recoveries of post-ischemia LVDP and RPP were expressed as a percentage of the pre-ischemic values during equilibration. To determine infarct size, hearts were perfused with 1% 2,3,5-triphenyltetrazolium chloride (TTC) following reperfusion and then incubated in TTC for 30 min at 37 °C. Hearts were fixed in 10% formaldehyde, and infarct size was expressed as a percentage of total area of cross-sectional slices.

2.4. Identification of protein SNO with 2D CyDye-maleimide DIGE

Hearts were snap frozen in liquid nitrogen after 15 min of Langendorff perfusion. Total heart homogenates were then prepared in the dark to prevent the cleavage of S-nitrosothiols, as previously described by Sun et al. [26]. Crude heart homogenate was obtained by grinding the heart into powder in liquid nitrogen and homogenizing in 1.5 ml buffer containing in mmol/l: 300 sucrose, 250 HEPES-NaOH pH 7.8, 1 EDTA, 0.1 neocuproine, and an EDTA-free protease inhibitor tablet per 10 ml (Roche Diagnostics Corporation, Indianapolis, IN) added just before use. Protein concentrations of the total homogenates were determined using the Bradford assay.

To assess differences in protein S-nitrosylation (SNO) using two-dimensional DIGE, we used a modified biotin switch protocol [27] with