Original Paper

17 β -Estradiol and/or Estrogen Receptor β Attenuate the Autophagic and Apoptotic Effects Induced by Prolonged Hypoxia Through HIF-1 α -Mediated BNIP3 and IGFBP-3 Signaling Blockage

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Key Words

17 β -estradiol • Estrogen receptor β • Hypoxia • Autophagy • Cardiac apoptosis

Abstract

Background/Aims: The risk of heart disease is higher in males than in females. However, this advantage of females declines with increasing age, presumably a consequence of decreased estrogen secretion and malfunctioning of the estrogen receptor. We previously demonstrated that 17 β -estradiol (E2) prevents cardiomyocyte hypertrophy, autophagy and apoptosis via estrogen receptor α (ER α), but the effects of ER β on myocardial injury remained elusive. The present paper thus, investigated the cardioprotective effects of estrogen (E2) and ER β against hypoxia-induced cell death. **Methods:** Transient transfection of Tet-On ER β gene construct was used to overexpress ER β in hypoxia-treated H9c2 cardiomyoblast cells. **Results:** Our data revealed that IGF1R, Akt phosphorylation and Bcl-2 expression are enhanced by ER β in H9c2 cells. Moreover, ER β overexpression reduced accumulation of hypoxia-related proteins, autophagy-related proteins and mitochondria-apoptotic proteins and enhanced the protein levels of Bcl-2, pAkt and Bad under hypoxic condition. In neonatal rat ventricular myocytes (NRVMs), we observed that hypoxia induced cell apoptosis as measured by TUNEL staining,

and E2 and/or ER β could totally abolish hypoxia-induced apoptosis. The suppressive effects of E2 and/or ER β in hypoxia-treated NRVMs were totally reversed by ER antagonist, ICI. Taken together, E2 and/or ER β exert the protective effect through repressed hypoxia-inducible HIF-1 α , BNIP3 and IGFBP-3 levels to restrain the hypoxia-induced autophagy and apoptosis effects in H9c2 cardiomyoblast cells. **Conclusion:** The results suggest that females probably could tolerate better prolonged hypoxia condition than males, and E2/ER β treatment could be a potential therapy to prevent hypoxia-induced heart damage."

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Introduction

Heart disease remains the most common cause of death worldwide over the past 30 years. Ischemic heart disease (IHD) is predicted to become the leading global cause of disease burden by 2020 [1]. Considerable data show that the morbidity rate from heart attack or coronary heart disease (CHD) is 2.4-fold higher in men than in women during age from 45-64 years and the gender gap narrows to 1.34 fold after age from 65-94 years [2]. This indicates that pre-menopausal females have lower risk of CHD than males, but post-menopausal females gradually lose this superiority. Therefore, hormone specific-effects may play an important role in females. Gender differences in the cardiovascular system have largely been ascribed to the effects of sexual steroid hormones such as estrogen. Estrogen produced by ovaries, testis or adrenal is essential in both male and female for a variety of physiologic processes and is classified into estrone (E1), 17 β -estradiol (E2) and estriol (E3). Moreover, 17 β -estradiol (E2), the abundant circulating form of estrogen in pre-menopausal females, is the most potent and predominant estrogen in human, but lower levels of E1 and E3 are also present. Estrogen has been identified as the effector of multiple functions including cell growth, differentiation and development of reproductive tissues. It is also thought to have a protective role on regulation of bone density, central nervous and cardiovascular systems [3]. In addition, estrogen-replacement therapy contributes to a low incidence of heart disease after menopause [4]. 17 β -estradiol (E2) has been shown to reduce cardiomyocyte apoptosis through activating phospho-inositide-3-kinase (PI3K)/Akt signaling in ovariectomized rats [5]. Previous studies in this lab also demonstrated that 17 β -estradiol (E2) could prevent ovariectomy-induced cardiac hypertrophy, Fas-dependent and mitochondria-dependent apoptotic pathways in rat models [6, 7].

According to the above findings, nearly all of the biological effects of estrogens are mediated by two distinct estrogen receptors (ERs), ER α and ER β [8]. ERs belong to a large superfamily of steroid/thyroid hormone nuclear receptors [9]. These ligand-regulated transcription factors that regulate the expression of estrogen-responsive genes share six structural and functional domains designated as A-F region [3, 8, 10]. The A/B domain contains activation functional domain-1 (AF-1). The C and D domains correspond to the DNA binding domain (DBD) and the hinge region, respectively. The E region includes a second activation functional domain-2 (AF-2) and an overlapping ligand binding domain (LBD). The F domain, located at the extreme carboxyl terminus, is regarded as a modulatory in ER activity. Importantly, the AF-1 domain mediated by phosphorylation is ligand-independent, whereas the AF-2 domain modulated by ligand-induced changes in receptor conformation is ligand-dependent [10, 11]. ER α and ER β possess similar binding affinities for E2 and their cognate DNA binding site (estrogen response element, ERE), which is likely caused by the high degree of sequence homology in their LBD and DBD [12]. However, Their A/B domain is exhibiting only an 18% identity between ER α and ER β [13, 14]. In some organs, ER α and ER β are expressed at similar levels, sometimes in different cell types within the same organ, whereas in others, one or the other subtype predominates. ER α is abundantly expressed in the uterus, prostate (stroma), ovary (theca cells), testes (Leydig cells), epididymis, bone, breast, liver, kidney, white adipose tissue, and various regions of the brain. ER β is dominantly

expressed in the colon, prostate (epithelium), testis, ovary (granulosa cells), bone marrow, salivary gland, vascular endothelium, lung, bladder, and certain regions of the brain. However, not only ER α but also ER β are distributed in the cardiovascular system [15].

We have previously demonstrated that 17 β -estradiol and/or ER α exert cardioprotective effects by suppressing JNK1/2-NF κ B-mediated LPS-induced TNF α expression and cardiomyocyte apoptosis via Akt activation [16]. Further unpublished studies in our lab also indicate that E2 and/or ER α could act against protein phosphatase 2A (PP2A)-induced cardiac hypertrophy and BNIP3-induced cardiac autophagy and apoptosis. However, effects of ER β on pathological conditions in heart are still unclear. Many retrospective studies point out that estrogen could attenuate pressure overload-induced cardiac remodeling and apoptosis and Angiotensin II (AngII)-produced cardiac hypertrophy and fibrosis modulated by ER β [17, 18]. These findings suggest that ER β also may play an important role in cardioprotection.

All the above findings show that E2 and/or ER β can inhibit apoptosis through activation of Akt, but there is no evidence about ER β regulating autophagy. Our studies aim to find out the regulatory mechanisms of E2 and/or ER β on hypoxia-induced autophagy and apoptosis. Two independent systems on ER β overexpression are used as the transient transfection and the Tet-On gene expression system in this present study.

Materials and Methods

Construct Tet-On ER β gene expression system

The Tet-On gene expression system belongs to a high-level gene expression using the regulator and response plasmids to establish a double-stable Tet cell line. The pTet-On regulator plasmid was based on a "reverse" Tet repressor (rTetR) which was converted by VP16 activation domain from a transcriptional repressor to a transcriptional activator forming a hybrid protein known as the reverse tetracycline-controlled transactivator (rtTA). The pTRE2-ER β response plasmid which was fused by pTRE2hyg-Luc plasmid and ER β cDNA through the restriction enzyme digestion in 5' cutting site of BamHI and 3' cutting site of Sall expressed ER β under the control of tetracycline-response element (TRE). Briefly, the parental H9c2 cells (rat embryonic cardiac myoblast; ATCC, VA, USA) were transfected with pTet-On plasmids containing neomycin-resistance genes that constitutively encoded rtTA proteins and pTRE2-ER β plasmids including hygromycin-resistance genes which expressed ER β proteins. In this Tet-On ER β system, rtTA protein binds to TRE and activates ER β transcription in response to doxycycline (Dox) in a precise and dose-dependent manner. The stable-clone selection was done in the presence of 200 μ g/ml G418 and 100 μ g/ml hygromycin B.

Cell culture

Wild-type or tet-on ER β H9c2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, MO, USA) supplemented with 10% CCS (HyClone, UT, USA), 2 mM glutamine, 1 mM pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin. Experiments were done in triplicates and for each experiment, H9c2 cardiomyoblast cells were used during 10 passages and then placed in a hypoxia chamber (NexBiOxy, Hsinchu, Taiwan) inside a humidified incubator (Thermo, NY, USA). This chamber was filled with a gas mixture (95% N₂ and 5% CO₂) at 37 °C for indicated time, and calibrated in 1% oxygen concentration by an oxygen analyzer (NexBiOxy). The control cells were maintained in normoxia (21% O₂-5% CO₂) at 37 °C. Before normoxia or hypoxia treatment for 24 h, 17 β -estradiol (E2, Sigma-Aldrich) or Dox (Clontech, CA, USA) was added 1 h and ER antagonist ICI 182780 (TOCRIS, Bristol, UK) was added 2 h in this study.

ER β overexpression through transient transfection

Cells with 50% confluence were replaced into fresh culture medium containing serum 2 h before transient transfection, and then plasmids of pCMV5-ER β were transfected in the cells for 24 h using PureFection™ Nanotechnology-based Transfection Reagent (System Biosciences, CA, USA) following the manufacturer's protocol. In each experiment, the efficiency of gene overexpression was measured by three independent Western blot analyses.

Western blot

Cells were lysed in 50 mM Tris-base (pH 7.4), 0.5 M NaCl, 1 M ethylenediamine tetraacetic acid (EDTA), 1 mM beta-mercaptoethanol (BME), 1% NP-40, 10% glycerol, IGEPAL CA-630 (Sigma-Aldrich) and protease inhibitor cocktail tablets (Roche, Mannheim, Germany) for 30 min and spun down at 12,000 rpm for 30 min, and then supernatants were collected for western blot analysis. The cell lysates were quantified by Bradford assays (Bio-Rad, CA, USA) and 30 µg of extracted proteins for each sample was separated by 8% and 12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). Nonspecific protein binding was blocked in Tris-buffered saline Tween-20 (TBS-T) containing 5% skim-milk for 1 h. The membranes were blotted with 1:1000 diluted primary antibodies against ERβ (Santa Cruz, CA, USA), p^{Y1161}IGF1R (Abcam, MA, USA), IGF1R (Abcam), p^{S473}-Akt (Cell Signaling, MA, USA), Akt1, Bcl-2, IGFBP-3, Bax, Bak, cytochrome c (all obtained from Santa Cruz), HIF-1α (Abcam), BNIP3, LC3B, p^{S112}-Bad, Atg7, Atg5, cleaved caspase-9, cleaved caspase-3 (all obtained from Cell Signaling), α-tubulin (Santa Cruz) or β-actin (Santa Cruz) at 4 °C overnight and incubated with secondary antibodies in RT for 1 h. Antibody interactions were visualized with enhanced chemiluminescence (ECL) horseradish peroxidase (HRP) substrate (Millipore). The densitometric analysis of protein level was performed by LAS 3000 imaging system (FUJIFILM, Tokyo, Japan).

Primary cardiomyocyte culture

Neonatal rat ventricular cardiomyocytes (NRVMs) were isolated and cultured using a commercially available Neonatal Rat Cardiomyocyte Isolation Kit (Cellutron Life Technology, MD, USA) according to the manufacturer's guidelines. In brief, hearts from one-day-old newborn Sprague-Dawley rats (BioLASCO, Taipei, Taiwan) were separated, the ventricles were pooled, and ventricular cells were released with digestion buffer at 37 °C. Ventricular cardiomyocytes were grown in NS medium (Cellutron Life Technology) supplemented with 10% fetal bovine serum (FBS). The experiments were performed in triplicates.

TUNEL assay and DAPI staining

NRVMs apoptosis was detected by in situ terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) using the In Situ Cell Death Detection Kit, Fluorescein (Roche), as indicated by the manufacturer. Hypoxia-treated cells after ERβ overexpression cultured in 24-well plates were fixed by 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 (TEDIA, NV, USA) in 0.1% sodium citrate for 2 min. After cellular nuclei were stained by DAPI (Sigma-Aldrich) (blue), cells with TUNEL-positive nuclei (green) were detected by fluorescence microscopy (Olympus, Tokyo, Japan). Three independent experiments were then averaged and statistically analyzed.

Statistical analysis

Quantitative data are shown as the mean ± SD corresponding to three or more replicates. Values are means ± S.E.M. Comparisons among the groups were carried out using Kruskal-Wallis one-way ANOVA with P < 0.05 considered to be statistically significant

Results

ERβ overexpression enhances specific IGF1R and Akt phosphorylation in both wild-type and Tet-On H9c2 cardiomyoblast cells

To examine whether ERβ could regulate the IGF1R/PI3K/Akt survival pathway, ERβ overexpression by transient transfection in H9c2 cells was used. Western blots indicated that ERβ expression, phosphorylation of IGF1R and Akt and protein level of Bcl-2 were significantly increased in an ERβ-dependent manner (Fig. 1A). Moreover, to identify whether ERβ expression is inducible in Tet-On H9c2 cells and confirm the prosurvival effect on transient transfection, we applied doxycycline (Dox) in Tet-On H9c2 cells. The results showed that ERβ expression is inducible and phosphorylation levels of IGF1R and Akt correlates with the level of transient transfection in a dose- and time-dependent manner (Fig. 1B and C). The data indicates that ERβ promotes myocardial survival via activation of IGF1R and Akt phosphorylation.

Fig. 1. Effect of ERβ on the phosphorylation of IGF1R and Akt survival factors in WT- and Tet-On ERβ H9c2 cardiomyoblast cells. IGF1R and Akt phosphorylation measured by Western blot analysis of total cell lysates from wild-type (WT) and Tet-On ERβ H9c2 cells. (A) ERβ plasmids were transiently transfected with indicating doses (1, 5 and 10 μg) for 24 h in H9c2 cells. ERβ overexpression significantly increased IGF1R and Akt phosphorylation and Bcl-2 protein level in a dose-dependent manner. (B and C) Tet-On ERβ H9c2 cells were treated with 1 μg/ml doxycycline (Dox) for up to 24 h and indicating doses (0.5, 1 and 2 μg/ml) for 24 h, separately. ERβ was induced by Dox which significantly enhanced IGF1R and Akt phosphorylation in a time- and a dose-dependent manner. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. normoxia-treated cells.

ERβ overexpression strongly promotes phosphorylation of Bad at S112 and Akt at S473 and suppresses both hypoxia-induced increase of HIF-1α, IGFBP-3 and BNIP3 to further reduce the expression of autophagic and apoptotic proteins in both wild-type and Tet-On H9c2 cardiomyoblast cells

Next, we further investigated whether ERβ overexpression could suppress hypoxia-induced autophagic and apoptotic pathways in H9c2 cells. We observed that the increase in the proteins such as HIF-1α, IGFBP-3, BNIP3 and LC3-II induced by hypoxia were significantly attenuated by ERβ overexpression (Fig. 2A). Moreover, the suppression of Akt and Bad phosphorylation under hypoxia was rescued by ERβ overexpression (Fig. 2A). In addition, we also used the Tet-On ERβ expression system to confirm the above results. Western blots revealed that Dox treatment not only reduces hypoxia-related proteins expression but also decreases the protein levels of Atg7, Atg5, Bax, Bak, Bcl-2, cytochrome c and cleaved caspase-9 (Fig. 2B). These results indicate that ERβ has a cardioprotective effect on hypoxia-induced autophagy and apoptosis by abolishing hypoxia-related proteins and enhancing the survival proteins.

Fig. 2. Roles of ERβ on hypoxia-induced autophagy and mitochondria-dependent apoptosis in WT- and Tet-On ERβ H9c2 cells. (A) Western blot was used to analysis total cell lysates from WT-H9c2 cells were transfected with ERβ plasmids by indicating amounts (5 and 10 μg) for 6 h before being exposed to hypoxia (1% O₂-5% CO₂) for 24 h. ERβ overexpression significantly attenuated the protein levels of HIF-1α, IGFBP-3 and BNIP3 and the processing of LC3-II, and enhanced phosphorylation of Akt at S473 and Bad at S112 under hypoxic condition. (B) Tet-On ERβ H9c2 cells were treated with Dox indicating doses (1 and 2 μg/ml) for 1 h before being exposed to hypoxia (1% O₂-5% CO₂) for 24 h. ERβ significantly suppressed hypoxia-induced proteins expression such as HIF-1α, IGFBP-3, BNIP3 (monomer forms), Atg7, Atg5, Bax, Bak, cytochrome c and cleaved caspase-9 in a dose-dependent manner. ***P<0.001 vs. normoxia-treated cells. ##P<0.01 and ###P<0.001 vs. hypoxia-treated cells.

E2 and/or ERβ overexpression significantly activate Akt phosphorylation and decrease the hypoxia-induced increase of BNIP3, LC3-II, Bak and cleaved caspase-3 in wild-type H9c2 cardiomyoblast cells

We further treated E2, ERβ or E2 plus ERβ to study whether E2 and/or ERβ could have a protective effect on hypoxia-induced cell death in H9c2 cells. We found that the increase

Fig. 3. Effects of E2 and/or ERβ on hypoxia-induced autophagy and mitochondria-dependent apoptosis in WT H9c2 cells. H9c2 cells were transiently transfected with 5 μg ERβ for 24 h, followed by the treatment of E2 (10⁻⁸ M) alone, E2 plus ERβ for 1 h, and then were exposed to hypoxia (1% O₂-5% CO₂) for 24 h. E2 and/or ERβ significantly decreased hypoxia-induced LC3-II processing and the apoptotic proteins expression of BNIP3, Bak and cleaved caspase-3, and strongly enhanced Akt phosphorylation. ***P*<0.01 and ****P*<0.001 vs. normoxia-treated cells. #*P*<0.05, ##*P*<0.01 and ###*P*<0.001 vs. hypoxia-treated cells.

amounts of proteins such as BNIP3, Bak, LC3-II and cleaved caspase-3 induced by hypoxia were significantly attenuated by E2 or ERβ (Fig. 3). Moreover, ERβ overexpression with or without E2 enhanced Akt phosphorylation after hypoxia treatment (Fig. 3). The data suggests that E2 prevents hypoxia-induced cardiomyoblast autophagy and apoptosis via ERβ.

E2 and/or ERβ overexpression attenuate hypoxia-induced apoptosis, but ER antagonist ICI totally abolishes the cardioprotective properties of E2 and/or ERβ in NRVMs

In order to confirm whether ERβ really plays an important role in providing cardioprotection against hypoxia-induced cellular damage in myocardial cells we used inhibitor assays to silence ERs. We found that E2 alone, ERβ overexpression and E2 plus ERβ indeed attenuated TUNEL-positive cells during hypoxia. However, ICI, a ERs inhibitor, strongly reversed the ERβ effect in hypoxia-treated NRVMs (Fig. 4).

Fig. 4. Roles of E2 and/or ER β on hypoxia-induced cell death in neonatal rat ventricular myocytes (NRVMs). Cell apoptosis stained by TUNEL assay from NRVMs, which were transfected with 1 μ g ER β for 24 h, followed by the treatment of E2 (10^{-8} M) alone, E2 plus ER β for 1 h after ER inhibitor (ICI, 10^{-6} M) treatment for 1 h, and then were exposed to hypoxia (1% O $_2$ -5% CO $_2$) for 24 h. Representative fluorescent images of TUNEL (green) and DAPI (blue) are shown. Hypoxia indeed increased TUNEL-positive cells, and E2 and/or ER β effectively suppressed the hypoxic effect. However, the E2 and/or ER β effects were totally reversed by ICI treatment. Data are presented as the mean $\pm$ SD (n=3). ** P <0.01 and *** P <0.001 vs. normoxia-treated cells. ## P <0.01 and ### P <0.001 vs. hypoxia-treated cells. \$ P <0.05 and \$\$\$ P <0.001 vs. hypoxia plus E2-treated cells. & P <0.05 and && P <0.01 vs. hypoxia plus ER β -treated cells. Φ P <0.05 vs. hypoxia plus E2/ER β -treated cells.

Discussion

The PI3K/Akt pathway is known to be involved in the anti-apoptotic effects of certain stimuli and plays a central role in cellular survival in many different cell types [19]. Akt activation by growth factors is thought to modulate the anti-apoptotic effects and to contribute to the cardioprotection and cell survival [20]. Meanwhile, it is also known that females have higher nuclear localization of phosphorylated-Akt and higher Akt activity in myocardium [21]. Interestingly, E2 has demonstrated to enhance the activation of Akt and to improve survival in murine cardiomyocytes [5, 7, 21]. More studies have identified that ER α specifically mediate the E2 induced activation of PI3K/Akt signaling pathway in vascular endothelial cells [22-24]. Our laboratory previously emphasized that E2 and ER α induce specific Akt and ERK1/2 phosphorylation [16]. In the present study, we focused on the question whether ER β is also involved in the PI3K/Akt survival pathway. The data presented here support that ER β overexpression not only enhanced Akt phosphorylation but also activated IGF1R phosphorylation and increased anti-apoptotic Bcl-2 protein expression (Fig. 1).

In animal and human studies, cardiomyocyte apoptosis had been presented within both the infarct and peri-infarct zones after coronary occlusion [25-31]. Although the role of E2/

Fig. 5. E2 and/or ER β expression provide cardio protection against HIF-1 α -mediated hypoxia. Schematic representation shows that E2 and/or ER β expression inhibit HIF-1 α -mediated hypoxia-induced autophagy and myocardial cell apoptosis through activation of Akt.

ER β in cardiomyocyte survival in response to myocardial hypoxia is not well understood, estrogen had been shown to reduce infarct size in ischemia-reperfusion injury [32, 33]. However, E2/ER β involved mechanism of protection against cardiomyocyte apoptosis that is critical in reducing myocardial injury is not clear yet. Moreover, our previous results have revealed that prolonged hypoxia suppresses IGF1R/PI3K/Akt involved myocardial survival pathway through HIF-1 α -IGFBP-3-dependent signaling and enhances cardiomyocyte autophagic and apoptotic effects mainly via FoxO3a-induced BNIP3 expression. In the present work we further evaluated whether E2 and/or ER β suppresses the hypoxia-induced autophagy and apoptosis. All our data indicate that E2, ER β and E2/ER β have the inhibitory effect on cell apoptosis of NRVMs induced by prolonged-hypoxia (Fig. 4). Moreover, the results were further confirmed from the ER β inhibition assay using the ER β antagonist ICI (Fig. 4). These results strongly suggest E2/ER β as an effective therapy for myocardial apoptosis induced by prolonged hypoxia.

The incidence and mortality of coronary heart disease are very low in pre-menopausal females but significantly higher in pro-menopausal females, indicating estrogen may act as a protector in cardiovascular system. However, results from the Women's Health Initiative suggest that estrogen therapy used in post-menopausal females has no beneficial impact against the development of cardiomyopathy such as arteriosclerotic coronary artery disease and myocardial infarction [34]. The controversy may be explained by the findings that estrogen prevents the development of early atherosclerotic lesions by reducing lipid deposits when hormone therapy is initiated in the premenopausal period or before the development of atherosclerosis [16]. However, estrogen can promote MMP expression, inflammatory activation and plaque instability once atherosclerosis has been established [34]. However, these previous studies mainly focused on vasculature and differ from the processes being explored in our study. So far, the mechanism of ER β to reduce hypoxia-induced apoptosis and the role of IGF1R/PI3K/Akt myocardial survival pathway in cardiomyocytes are not known. Our results support the hypothesis that ER β not only attenuate hypoxia-induced autophagy and apoptosis by suppressing HIF-1 α and the downstream BNIP3 and IGFBP-3 signaling pathways, but also mediate the activation of IGF1R/PI3K/Akt myocardial survival pathway (Fig. 2). These findings strongly suggest that the ER β pathway could be an effective therapeutic target in myocardial apoptosis induced by prolonged hypoxia.

Furthermore, the present study clearly shows that the cardioprotective effects and mechanisms of E2 and ER β involve the inhibition of hypoxia-induced HIF-1 α and downstream BNIP3 and IGFBP3-dependent apoptotic responses in myocardial cells (Fig. 5). These findings may explain why females have lower rates of mortality due to heart dysfunction and less acute inflammatory responses than males, and also why females probably could tolerate prolong hypoxia condition than males.

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Disclosure Statement

The authors declare that they have no conflict of interest.

References

- 1 Lopez AD, Murray CC: The global burden of disease, 1990-2020. *Nat Med* 1998;4:1241-1243.
- 2 Mosca L, Barrett-Connor E, Wenger NK: Sex/gender differences in cardiovascular disease prevention: What a difference a decade makes. *Circulation* 2011;124:2145-2154.
- 3 Osborne CK, Zhao H, Fuqua SA: Selective estrogen receptor modulators: Structure, function, and clinical use. *J Clin Oncol* 2000;18:3172-3186.
- 4 Ylikorkala O: Hrt as secondary prevention of cardiovascular disease. *Maturitas* 2004;47:315-318.
- 5 Patten RD, Pourati I, Aronovitz MJ, Baur J, Celestin F, Chen X, Michael A, Haq S, Nuedling S, Grohe C, Force T, Mendelsohn ME, Karas RH: 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/akt signaling. *Circ Res* 2004;95:692-699.
- 6 Liou CM, Yang AL, Kuo CH, Tin H, Huang CY, Lee SD: Effects of 17beta-estradiol on cardiac apoptosis in ovariectomized rats. *Cell Biochem Funct* 2010;28:521-528.
- 7 Wu CH, Liu JY, Wu JP, Hsieh YH, Liu CJ, Hwang JM, Lee SD, Chen LM, Chang MH, Kuo WW, Shyu JC, Tsai JH, Huang CY: 17beta-estradiol reduces cardiac hypertrophy mediated through the up-regulation of pi3k/akt and the suppression of calcineurin/nf-at3 signaling pathways in rats. *Life Sci* 2005;78:347-356.
- 8 Osborne CK: Steroid hormone receptors in breast cancer management. *Breast Cancer Res Treat* 1998;51:227-238.
- 9 Olefsky JM: Nuclear receptor minireview series. *J Biol Chem* 2001;276:36863-36864.
- 10 Tsai MJ, O'Malley BW: Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 1994;63:451-486.
- 11 Tora L, White J, Brou C, Tasset D, Webster N, Scheer E, Chambon P: The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* 1989;59:477-487.
- 12 Coleman KM, Dutertre M, El-Gharbawy A, Rowan BG, Weigel NL, Smith CL: Mechanistic differences in the activation of estrogen receptor-alpha (ER alpha)- and ER beta-dependent gene expression by camp signaling pathway(s). *J Biol Chem* 2003;278:12834-12845.
- 13 Delaunay F, Pettersson K, Tujague M, Gustafsson JA: Functional differences between the amino-terminal domains of estrogen receptors alpha and beta. *Mol Pharmacol* 2000;58:584-590.
- 14 Pearce ST, Jordan VC: The biological role of estrogen receptors alpha and beta in cancer. *Crit Rev Oncol Hematol* 2004;50:3-22.
- 15 Nilsson S, Gustafsson JA: Estrogen receptors: Therapies targeted to receptor subtypes. *Clin Pharmacol Ther* 2011;89:44-55.
- 16 Liu CJ, Lo JF, Kuo CH, Chu CH, Chen LM, Tsai FJ, Tsai CH, Tzang BS, Kuo WW, Huang CY: Akt mediates 17beta-estradiol and/or estrogen receptor-alpha inhibition of lps-induced tumor necrosis factor-alpha expression and myocardial cell apoptosis by suppressing the jnk1/2-nfkappab pathway. *J Cell Mol Med* 2009;13:3655-3667.
- 17 Fliegner D, Schubert C, Penkalla A, Witt H, Kararigas G, Dworatzek E, Staub E, Martus P, Ruiz Noppinger P, Kintscher U, Gustafsson JA, Regitz-Zagrosek V: Female sex and estrogen receptor-beta attenuate cardiac remodeling and apoptosis in pressure overload. *Am J Physiol Regul Integr Comp Physiol* 2010;298:R1597-1606.

- 18 Pedram A, Razandi M, Lubahn D, Liu J, Vannan M, Levin ER: Estrogen inhibits cardiac hypertrophy: Role of estrogen receptor-beta to inhibit calcineurin. *Endocrinology* 2008;149:3361-3369.
- 19 Datta SR, Brunet A, Greenberg ME: Cellular survival: A play in three acts. *Genes Dev* 1999;13:2905-2927.
- 20 Chao W, Matsui T, Novikov MS, Tao J, Li L, Liu H, Ahn Y, Rosenzweig A: Strategic advantages of insulin-like growth factor-I expression for cardioprotection. *J Gene Med* 2003;5:277-286.
- 21 Camper-Kirby D, Welch S, Walker A, Shiraishi I, Setchell KD, Schaefer E, Kajstura J, Anversa P, Sussman MA: Myocardial akt activation and gender: Increased nuclear activity in females versus males. *Circ Res* 2001;88:1020-1027.
- 22 Haynes MP, Sinha D, Russell KS, Collinge M, Fulton D, Morales-Ruiz M, Sessa WC, Bender JR: Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the pi3-kinase-akt pathway in human endothelial cells. *Circ Res* 2000;87:677-682.
- 23 Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK: Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-oh kinase. *Nature* 2000;407:538-541.
- 24 Hisamoto K, Ohmichi M, Kurachi H, Hayakawa J, Kanda Y, Nishio Y, Adachi K, Tasaka K, Miyoshi E, Fujiwara N, Taniguchi N, Murata Y: Estrogen induces the akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. *J Biol Chem* 2001;276:3459-3467.
- 25 Kang PM, Izumo S: Apoptosis and heart failure: A critical review of the literature. *Circ Res* 2000;86:1107-1113.
- 26 van Eickels M, Patten RD, Aronovitz MJ, Alsheikh-Ali A, Gostyla K, Celestin F, Grohe C, Mendelsohn ME, Karas RH: 17-beta-estradiol increases cardiac remodeling and mortality in mice with myocardial infarction. *J Am Coll Cardiol* 2003;41:2084-2092.
- 27 Zhao ZQ, Nakamura M, Wang NP, Wilcox JN, Shearer S, Ronson RS, Guyton RA, Vinten-Johansen J: Reperfusion induces myocardial apoptotic cell death. *Cardiovasc Res* 2000;45:651-660.
- 28 Palojoki E, Saraste A, Eriksson A, Pulkki K, Kallajoki M, Voipio-Pulkki LM, Tikkanen I: Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats. *Am J Physiol Heart Circ Physiol* 2001;280:H2726-2731.
- 29 Saraste A, Pulkki K, Kallajoki M, Henriksen K, Parvinen M, Voipio-Pulkki LM: Apoptosis in human acute myocardial infarction. *Circulation* 1997;95:320-323.
- 30 Olivetti G, Quaini F, Sala R, Lagrasta C, Corradi D, Bonacina E, Gambert SR, Cigola E, Anversa P: Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart. *J Mol Cell Cardiol* 1996;28:2005-2016.
- 31 Abbate A, Biondi-Zoccai GG, Bussani R, Dobrina A, Camilot D, Feroce F, Rossiello R, Baldi F, Silvestri F, Biasucci LM, Baldi A: Increased myocardial apoptosis in patients with unfavorable left ventricular remodeling and early symptomatic post-infarction heart failure. *J Am Coll Cardiol* 2003;41:753-760.
- 32 Hale SL, Birnbaum Y, Kloner RA: Estradiol, administered acutely, protects ischemic myocardium in both female and male rabbits. *J Cardiovasc Pharmacol Ther* 1997;2:47-52.
- 33 Node K, Kitakaze M, Kosaka H, Minamino T, Funaya H, Hori M: Amelioration of ischemia- and reperfusion-induced myocardial injury by 17beta-estradiol: Role of nitric oxide and calcium-activated potassium channels. *Circulation* 1997;96:1953-1963.
- 34 Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J: Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the women's health initiative randomized controlled trial. *JAMA* 2002;288:321-333.