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

Dennis Jine-Yuan Hsieh\textsuperscript{a,b} Wei-Wen Kuo\textsuperscript{c} Yi-Ping Lai\textsuperscript{d} Marthandam Asokan Shibu\textsuperscript{d} Chia-Yao Shen\textsuperscript{n} Peiying Pai\textsuperscript{f} Yu-LanYeh\textsuperscript{g} Jing-Ying Lin\textsuperscript{h} Vijaya Padma Viswanadha\textsuperscript{1} Chih-Yang Huang\textsuperscript{d,j,k}

\textsuperscript{a}School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, \textsuperscript{b}Department of Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, \textsuperscript{c}Department of Biological Science and Technology, China Medical University, Taichung, \textsuperscript{d}Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan, \textsuperscript{e}Department of Nursing, MeiHo University, Pingtung, Taiwan, \textsuperscript{f}Division of Cardiology, China Medical University Hospital, Taichung, Taiwan, \textsuperscript{g}Department of Cardiology, Changhua Christian Hospital, Changhua, \textsuperscript{h}Department of Nursing, Central Taiwan University of Science and Technology, Taichung, Taiwan; \textsuperscript{i}Department of Biotechnology, Bharathiar University, Coimbatore, India; \textsuperscript{j}Graduate Institute of Chinese Medical Science, China Medical University, Taichung, Taiwan; \textsuperscript{k}Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan

Key Words
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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 17β-estradiol (E2) prevents cardiomyocyte hypertrophy, autophagy and apoptosis via estrogen receptor α (ERα), but the effects of ERβ on myocardial injury remained elusive. The present paper thus, investigated the cardioprotective effects of estrogen (E2) and ERβ against hypoxia-induced cell death. Methods: Transient transfection of Tet-On ERβ gene construct was used to overexpress ERβ in hypoxia-treated H9c2 cardiomyoblast cells. Results: Our data revealed that IGF1R, Akt phosphorylation and Bcl-2 expression are enhanced by ERβ 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 ERβ could totally abolish hypoxia-induced apoptosis. The suppressive effects of E2 and/or ERβ in hypoxia-treated NRVMs were totally reversed by ER antagonist, ICI. Taken together, E2 and/or ERβ exert the protective effect through repressed hypoxia-inducible HIF-1α, 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/ERβ treatment could be a potential therapy to prevent hypoxia-induced heart damage."
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 ERα but also ERβ are distributed in the cardiovascular system [15].

We have previously demonstrated that 17β-estradiol and/or ERα exert cardioprotective effects by suppressing JNK1/2-NFκB-mediated LPS-induced TNFα expression and cardiomyocyte apoptosis via Akt activation [16]. Further unpublished studies in our lab also indicate that E2 and/or ERα could act against protein phosphatase 2A (PP2A)-induced cardiac hypertrophy and BNIP3-induced cardiac autophagy and apoptosis. However, effects of ERβ 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 ERβ [17, 18]. These findings suggest that ERβ also may play an important role in cardioprotection.

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

**Materials and Methods**

**Construct Tet-On ERβ 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-ERβ response plasmid which was fused by pTRE2hyg-Luc plasmid and ERβ cDNA through the restriction enzyme digestion in 5’ cutting site of BamHI and 3’ cutting site of SalI expressed ERβ 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-ERβ plasmids including hygromycin-resistance genes which expressed ERβ proteins. In this Tet-On ERβ system, rtTA protein binds to TRE and activates ERβ transcription in response to doxycycline (Dox) in a precise and dose-dependent manner. The stable-clone selection was done in the presence of 200 μg/ml G418 and 100 μg/ml hygromycin B.

**Cell culture**

Wild-type or tet-on ERβ 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 μg/ml streptomycin. Experiments were done in triplicates and for each experiment, H9c2 cells were cultured 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, 17β-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.

**ERβ 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-ERβ 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), p1161IGF1R (Abcam, MA, USA), IGF1R (Abcam), p473-Akt (Cell Signaling, MA, USA), Akt1, Bcl-2, IGFBP-3, Bax, Bak, cytochrome c (all obtained from Santa Cruz), HIF-1α (Abcam), BNIP3, LC3B, p112-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 ºC 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 ºC. 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.
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.
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*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...
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).
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 ERα specifically mediate the E2 induced activation of PI3K/Akt signaling pathway in vascular endothelial cells [22-24]. Our laboratory previously emphasized that E2 and ERα induce specific Akt and ERK1/2 phosphorylation [16]. In the present study, we focused on the question whether ERβ is also involved in the PI3K/Akt survival pathway. The data presented here support that ERβ 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/
ERβ 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/ERβ 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-1α-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 ERβ suppresses the hypoxia-induced autophagy and apoptosis. All our data indicate that E2, ERβ and E2/ERβ have the inhibitory effect on cell apoptosis of NRVMs induced by prolonged-hypoxia (Fig. 4). Moreover, the results were further confirmed from the ERβ inhibition assay using the ERβ antagonist ICI (Fig. 4). These results strongly suggest E2/ERβ 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 ERβ 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 ERβ not only attenuate hypoxia-induced autophagy and apoptosis by suppressing HIF-1α and the downstream BNIP3 and IGFBP3 signaling pathways, but also mediate the activation of IGF1R/PI3K/Akt myocardial survival pathway (Fig. 2). These findings strongly suggest that the ERβ pathway could be an effective therapeutical target in myocardial apoptosis induced by prolonged hypoxia.

Furthermore, the present study clearly shows that the cardioprotective effects and mechanisms of E2 and ERβ involve the inhibition of hypoxia-induced HIF-1α 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 prolonged hypoxia condition than males.
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Disclosure Statement

The authors declare that they have no conflict of interest.

References