Estrogen Attenuates Chronic Stress-Induced Cardiomyopathy by Adaptively Regulating Macrophage Polarizations via $\beta_2$-Adrenergic Receptor Modulation

Hongjian Hou$^{1,2,\ast}$, Gabriel Komla Adzika$^{1,\dagger}$, Qi Wu$^1$, Tongtong Ma$^1$, Yanhong Ma$^1$, Juan Geng$^1$, Mingjin Shi$^1$, Lu Fu$^1$, Ruqayya Rizvi$^3$, Zheng Gong$^4$ and Hong Sun$^{1,3,\ast}$

$^1$Department of Physiology, Xuzhou Medical University, Xuzhou, China, $^2$The College of Biology and Food, Shangqiu Normal University, Shangqiu, China, $^3$Xuzhou Medical University, Xuzhou, China, $^4$The School of Public Affairs and Governance, Silliman University, Dumaguete, Philippines

Clinical demographics have demonstrated that postmenopausal women are predisposed to chronic stress-induced cardiomyopathy (CSC) and this has been associated with the decrease of estrogen. Meanwhile, recent studies have implicated unsolved myocardial proinflammatory responses, which are characterized by enormous CD86+ macrophage infiltrations as an underlying disease mechanism expediting the pathological remodeling of the heart during chronic stress. However, we had previously demonstrated that estrogen confers cardioprotection via the modulation of cardiomyocytes $\beta_2$-adrenoceptors ($\beta_2$AR)-Gs/Gi pathways during stress to lessen the incidence of stress-induced cardiovascular diseases in premenopausal women. Intriguingly, macrophages express $\beta_2$AR profoundly as well; as such, we sought to elucidate the possibilities of estrogen modulating $\beta_2$AR-Gs/Gi pathway to confer cardioprotection during stress via immunomodulation. To do this, ovariectomy (OVX) and sham operations (Sham) were performed on female Sprague-Dawley (SD) rats. Two weeks after OVX, the rats were injected with 40 $\mu$g/kg/day of estradiol (E$_2$). Next, on day 36 after OVX, chronic stress was induced by a daily subcutaneous injection of 5 mg/kg/day of isoproterenol (ISO). The effect of E$_2$ on relevant clinical cardiac function indexes (LVSP, LVEDP, $+\frac{dp}{dt}$ and $-\frac{dp}{dt}$), myocardial architecture (cardiomyocyte diameter and fibrosis), $\beta_2$AR alterations, and macrophage (CD86+ and CD206+) infiltrations were assessed. In vitro, peritoneal macrophages (PM$_{8}$) were isolated from wild-type and $\beta_2$AR-knockout female mice. The PM$_{8}$ were treated with ISO, E$_2$, and $\beta_2$AR blocker ICI 118,551 for 24 h, and flow cytometric evaluations were done to assess their phenotypic expression. E$_2$ deficiency permitted the induction of CSC, which was characterized by cardiac dysfunctions, maladaptive myocardial hypertrophy, unresolved proinflammatory responses, and fibrosis. Nonetheless, E$_2$ presence/supplementation during stress averted all the aforementioned adverse effects of chronic stress while preventing excessive depletion of $\beta_2$AR. Also, we demonstrated
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β2AR Mediates Estrogenic Macrophage Polarization

INTRODUCTION

Similar to other cardiovascular diseases (CVDs), chronic stress-induced cardiomyopathy (CSC) has been clinically shown to be predominant in males of all age cohorts, while females are mostly predisposed to its occurrence during their menopausal period (Boese et al., 2017; Ndzie Noah et al., 2021). Recent attempts to elucidate the underlying disease mechanisms have revealed crucial roles played by estrogen during stress to sustain good cardiac health in premenopausal women, besides its reproductive functions (Mori et al., 2011; Boese et al., 2017).

Typically, the clinical manifestations of CSC patients are severe left ventricular (LV) diastolic dysfunction (LVDD) and systolic dysfunctions (LVSD) (Medeiros et al., 2014). The adverse structural remodeling includes excessive LV hypertrophy and massive interstitial fibrosis, which results in the stiffening of the myocardia and causes these cardiac dysfunctions. Also, recent studies have demonstrated that unresolved myocardial inflammatory responses characterized by the enormous influx of proinflammatory macrophages exacerbates CSC and aggravates adverse cardiac remodeling (Wilson et al., 2018; Scally et al., 2019).

Under physiological state, inotropic and chronotropic functions of the heart are mediated by β-adrenergic receptors (βARs) via G stimulatory protein (Gs), mainly β1AR and β2AR. However, hyperstimulation of the receptors during chronic stress results in the downregulation of β1AR mostly, while β2AR traffics the stimuli signal via G inhibitory protein (Gi) to prevent cardiac insults (Paur et al., 2012). The homologous desensitization of βARs which results in the downregulation is induced by G protein-coupled receptor kinases 2 (GRK2) phosphorylation and β-arrestin-1 recruitment (Adzika et al., 2019). Nonetheless, it was demonstrated in previous studies that estrogen ameliorates stress-induced cardiac insults by preventing excessive depletion of β2ARs and also facilitating a balance in the Gi/Gs signaling pathways (Cao et al., 2015; Hou et al., 2018). The estrogenic effects of sustaining β2AR activities during stress might be likely due to its inhibitory effects on GRK2 (Abraham et al., 2018). Intriguingly, β2AR are profoundly expressed on macrophages; hence, estrogen may indirectly modulate their activities and possibly their polarizations into proinflammatory (CD86+ macrophages) or anti-inflammatory (CD206+) macrophages in the myocardia. It is hypothesized that the possible exertion of the aforementioned estrogenic immunoregulation might prevent extensive pathological cardiac remodeling during chronic stress by subduing maladaptive myocardial hypertrophy, fibrosis, and proinflammatory responses.

Herein, we sought to explore the cardioprotective mechanisms employed by estrogen via immunomodulation to decrease the incidence of CSC in female rat models, as understanding these mechanisms will provide the basis for further translational research into preventing CSC in postmenopausal women.

MATERIALS AND METHODS

Experimental Animals and Models

The wild-type and β2AR knockout FVB female mice (donated by Professor Daniel Bernstein, Stanford University—United States) and adult female Sprague-Dawley (SD) rats (180–200 g) were used for the experiments (n = 4–8 rats/group). All standard animal house boundary protocols were observed. After ensuring the SD rats were in the same menstrual phase through vaginal mucus examination, bilateral ovariectomy (OVX) and sham surgeries were done as we previously described (Zhang et al., 2021).

As illustrated (Figure 1A), 2 weeks after ovariectomy, the rats were intraperitoneally injected with 40 μg/kg/day of estradiol (E2) (E2758; Sigma, St. Louis, MO, United States) for 31 days, as done previously (Zhang et al., 2021). Olive oils of equivalent amounts were administered as a placebo to the control groups. On day 36 after ovariectomy, chronic stress was induced in rats that were being treated with either E2 or the placebo by subcutaneous injections of isoproterenol (ISO) (160504; Sigma) at 5 mg/kg/day for 10 days, as previously done (Lin et al., 2016; Zhou et al., 2017). Also, the Sham surgery rats had similar ISO and placebo treatments. In total, in vivo experiments included the following six groups: (i) Sham group, (ii) OVX group, (iii) OVX + E2 group, (iv) Sham + ISO group, (v) OVX + ISO group, and (vi) OVX + ISO + E2 group.

The dosage of E2 employed was to mimic its physiological levels in rats, as we had demonstrated previously (Liu et al., 2012; Zhang et al., 2021). Also, rather than the high dosage of ISO used in acute stress models, as done previously (Youssef et al., 2021), a relatively milder dosage was used due to the prolonged duration (10 days) of the catecholamine treatment (Zhang et al., 2021).

Hemodynamic Experiment

After properly sedating rats (n = 7 rats/group), they were fixed in the supine position, and longitudinal incisions (about 2 cm in
length) were made in the mid-neck. By using blunt hemostatic forceps, the fascia and aponeurosis were separated to reveal 1–1.5 cm of the right common carotid artery. The distal end of the right common carotid artery was ligated, and the proximal end of the right common carotid artery was clamped with an arterial clamp. Next, an ophthalmic scissor was used to nick the artery (in a V-shaped), a heparin-filled catheter attached to a pressure transducer was carefully inserted into the left ventricle.
The left ventricular systolic and end-diastolic pressures and electrocardiography (ECG) were recorded with PowerLab data acquisition system (ADInstruments, North America, Colorado Springs, CO, United States).

**Histological Assessment of Myocardia**

Excised hearts ($n = 6$ hearts/group) were properly washed with prechilled PBS, blotted with filter paper, and fixed in 4% paraformaldehyde for more than 48 h. Next, the heart specimens were embedded in paraffin, sectioned at 4 µm thickness, and preserved for histological assessments.

The myocardial sectionings were deparaffinized before performing Masson’s trichrome (Maxim Biotechnologies, Fuzhou, China), hematoxylin and eosin (H&E), and immunohistochemical (IHC) staining as previously described (Zhang et al., 2021). The trichrome staining were done to ascertain the collagen volume fraction (CVF) while H&E staining were performed to discern cardiomyocyte diameters and help depict the extent of myocardial hypertrophy. Also, IHC staining with CD68 (Abcam, Cambridge, United Kingdom; ab955), CD86 (Bioss, Woburn, MA, United States; BS-1035R), and CD206 (Abcam; ab8918) was done to assess the extent of myocardial infiltrations of inflammatory cells.

Imaging of all stained sections were done at $×400$ magnification (IX 71, Olympus, Tokyo, Japan) and analyzed using ImageJ (1.53a version; National Institute of Health, Bethesda, MD, United States).

**Quantitative Real-Time PCR**

Trizol (Invitrogen Co., Carlsbad, CA, United States) was used to extract RNAs from homogenized myocardia ($n = 4$ hearts/group). After the normalization of RNA concentrations, cDNAs were synthesized using Revertrase ace® qPCR rt kit (Toyobo, Osaka, Japan). By using SYBR Green Master Mix (Vazyme Biotech, Nanjing, China), the following gene primers (Sangon Biotech, Shanghai, China) were used to evaluate mRNA expressions: (1) Tumor necrosis factor-alpha (TNF-α), Forward: GAAAGCTGATCCGAGATTTG; Reverse: CACGAGCAGGAATGAGAAG, (2) transforming growth factor-beta (TGF-β1), Forward: ATGGTGAGCCACAAGCGC; Reverse: GTCGCCACGTCTCTCCCCAGTCTC, (3) inducible nitric oxide synthase (iNOS), Forward: TCTTGAGCCAGTTGCACTTGT; Reverse: TAGGTGAGGCTTGGCCTGAGTG; (4) arginase 1 (Arg-1), Forward: CCTTGAGCGGTTGCTTCC; Reverse: CTGGCCACTGTCCTCCCGAAGTGC, (5) glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Forward: TCCTGTGCACCAACTGCTTAG; Reverse: AGTTGCCAGTGATGCGATGACT.

The $2^{-ΔΔCt}$ analysis method was used to evaluate the relative mRNA levels as described (Gold et al., 2012) and have been graphically presented as fold changes compared with the Sham group.

**Western Blotting**

Proteins were extracted from myocardial apexes ($n = 4$ hearts/group), treated with reducing agents, denatured at 100°C, and separated by gel electrophoresis as previously described (Hou et al., 2018). Next, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, blocked with 1% bovine serum albumin, and incubated in the following primary antibodies at 4°C overnight: ANP (1:1,000, Santa Cruz Biotechnology, Dallas, TX, United States; sc-515701), BNP (1:1,000, Santa Cruz Biotechnology; sc-271185), β2AR (1:1,000, Abcam; ab182136), GAPDH (1:4,000, ProteinTech, Manchester, United Kingdom; 10494-1-AP). Visualizations of immunoblots were done with enhanced chemiluminescence (Tanon, Shanghai, China). The protein bands were quantified and evaluated by the relative expressions with their GAPDH.

**Isolation and Characterization of Peritoneal Macrophages for in vitro Experiments**

Peritoneal macrophages (PMφ) ($n ≤ 2 \times 10^6$ cells) were harvested from wild-type (WT) and β2AR knockout (β2AR-KO) FVB female mice by using methods previously demonstrated (Ray and Dittel, 2010). In brief, the mice peritoneum were exposed under aseptic conditions. Five to 10 ml of prewarmed (37°C) 3% fetal bovine serum (FBS) were injected into the peritoneal cavity. Cell suspensions were collected after softly massaging for 5 min and centrifuged at 1,500 rpm for 10 min, and the obtained cell pellets were resuspended and cultured with 10% FBS at 37°C and 5% CO₂ for 48 h. Next, 24 h in vitro treatments of cultured PMφ included: ISO (10 μM), E2 (1 nM), and β2AR blocker ICI 118,551 (55 nM) (Figure 1B). These treatments were preceded by E2 pretreatments for 1 h, in groups where the estrogenic effects were to be ascertained.

The identification and subtyping of isolated PMφ after culturing or treatments were done by flow cytometry BD LSR II (BD Biosciences, San Jose, CA, United States). APC anti-F4/80 (123316; BioLegend, San Diego, CA, United States) and FITC anti-CD11b (123116; BioLegend, San Diego, CA, United States) and PE anti-CD206 (141706; BioLegend) antibodies were used to identify the macrophages while PerCP anti-CD86 (105028; BioLegend) and PE anti-CD206 (141706; BioLegend) antibodies were used to differentially identify M1 macrophages and M2 macrophages, respectively. Preparations of cultured or treated PMφ for flow cytometry were done as previously described (Zhu et al., 2017). Acquired data were analyzed with FlowJo software (v10; FlowJo LLC, Oregon, OR, United States).

**Statistical Analysis**

Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, United States). All data were presented as mean ± SEM and compared by two-way ANOVA. $p$-values < 0.05 were deemed statistically significant.

**RESULTS**

**Estrogen Deficiency Facilitates Weight Gains During Chronic Stress**

Analysis of the morphometrics of rats demonstrated that E2-deficient (OVX) rats gained significant body weights...
Estrogen Deficiency Aggravates Isoproterenol-Induced Cardiac Dysfunction

E₂ deficiency during chronic stress resulted in decreased heart rates (HR) in OVX rats. The supplementation of E₂ in OVX rats and the presence of E₂ in Sham rats prevented a significant decrease in HR during chronic stress (Figure 2A). Furthermore, the cardiac function index; LVSP, LVEDP, the rate of pressure development (+dp/dt), and the rate of pressure development decay (−dp/dt) were assessed to ascertain for any occurring dysfunctionalities. It was demonstrated that E₂ deficiency during chronic stress resulted in depressions in LVSP, LVEDP, +dp/dt, and −dp/dt. However, E₂ and E₂ prevented significant alterations in these cardiac function indexes during stress (Figures 2B–E).

Estrogen Deficiency Promotes Myocardial Hypertrophy and Fibrosis During Chronic Stress

To ascertain the impact of chronic stress on the myocardial architecture, H&E and trichrome staining were done to evaluate the extent of cardiomyocyte hypertrophy and interstitial collagen deposition, respectively. The measurements of cardiomyocyte diameters from H&E-stained myocardia across all groups demonstrated that, under physiological state, the deficiency of E₂ does not affect the cell sizes. However, E₂ deficiency (in OVX rats) during chronic stress permits excessive cardiomyocyte hypertrophy. Also, the obtained results showed that, while E₂ in general inhibited excessive cardiomyocyte hypertrophy during stress in both Sham and OVX + E₂ groups, E₂ in Sham (in Sham) exhibited much more potent antihypertrophic effects than E₂ in OVX + E₂ did (Figures 3A,B). Next, immunoblotting of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) depicted the maladaptive nature of the resulting cardiomyocyte hypertrophy when E₂ is deficient during stress. E₂ relatively decreased the expressions of both natriuretic peptides; whereas, E₂ only affected ANP upregulations (Figures 3C–F).

Assessed CVF demonstrated that myocardial interstitial fibrosis increases during chronic stress in OVX rats; however, the results trend showed that the presence of E₂ does ameliorate its severity but without statistical significance on comparing with OVX + E₂ + ISO and Sham + ISO groups (Figures 3G,H).

Estrogen Attenuates Maladaptive Myocardial Inflammatory Responses During Chronic Stress

Myocardial inflammation during chronic stress contributes to exaggerated cardiac remodeling (Hulsmans et al., 2018). Hence, we assessed the potentials of E₂ in exerting adaptive immunoregulation in the myocardia during stress. CD68-positive IHC staining demonstrated that, under physiological state, the amount of macrophages infiltrating the myocardia are slightly elevated when E₂ is deficient (in OVX rats).

Also, although CD68-positive cell infiltrations were generally increased during chronic stress, significant upregulations only resulted in OVX + ISO rats. The presence of E₂ and E₂ in Sham + ISO and OVX + E₂ + ISO, respectively, prevented enormous CD68-positive cell infiltration into the myocardia during stress (Figures 4A,B). Furthermore, by using CD86 and CD206 IHC staining, it is shown that majority of the inflammatory cells infiltrating the myocardia during stress when E₂ is deficient are CD86-positive (proinflammatory) cells, while CD206-positive (anti-inflammatory) cell infiltrations are significantly hampered. However, the contrast of this phenomenon is demonstrated by E₂ and E₂ in Sham + ISO and OVX + E₂ + ISO, respectively, during stress. The anti-inflammatory cell infiltrations are significantly increased while proinflammatory cell infiltrations were dampened in these groups. Also, it is observed that E₂ was potent than E₂ in the adaptive modulation of myocardial inflammatory cell infiltrations (Figures 4C–F). To validate the adaptive immunoregulation exerted by E₂, mRNAs of proinflammatory (TNF-α and iNOS) and anti-inflammatory (TGF-β1 and Arg-1) biomarkers were assessed from the myocardia. During chronic stress, E₂ deficiency (in OVX rats) permitted upregulations of TNF-α and iNOS while TGF-β1 and Arg-1 expressions were downregulated. Conversely, E₂ enhanced the expressions of TGF-β1 and Arg-1 and decreased TNF-α and iNOS levels (Figures 4G–J).

Estrogenic Adaptive Immunoregulation of Macrophage Polarization Involves Modulation of β₂AR Signaling Activities

In our previous studies (Hou et al., 2018), it was demonstrated that E₂ conferred cardioprotective effects via the modulation of β₂AR-Gα_/Gβγ-mediated signaling cascades during stress. Hence, to elucidate the underlying mechanism employed by E₂ to facilitate timely resolutions of myocardial proinflammatory responses, we again investigated the likely involvement of β₂AR since they are well expressed in both cardiomyocytes and macrophages. Immunoblotting results showed a significant decrease in β₂AR expression in OVX + ISO rats (Figures 5A,B). However, the extent of β₂AR downregulations in Sham + ISO and OVX + E₂ + ISO was relatively lower than OVX + ISO, which showed statistical significance when compared with OVX. Flowcytometry evaluations of PMs, isolated from WT and β₂AR-KO and treated with ISO (10 µM) and/or E₂ (1 nM) along with or without β₂AR blocker ICI 118,551...
FIGURE 2 | Estrogen deficiency permits cardiac dysfunction chronic stress. (A) Graphical representation of heart rates (HR). (B,C) Left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) recordings depict cardiac dysfunction in OVX + ISO rats. (D,E) Rate of pressure development (+ dp/dt) and the rate of pressure development decay (−dp/dt) further validate cardiac dysfunction in OVX + ISO rats. \( n = 7 \) rats/group. \( * p < 0.05, ** p < 0.01, \) and \( *** p < 0.001 \). Data are presented as mean ± SEM. Data were analyzed using two-way ANOVA and Bonferroni’s multiple comparisons test.

(55 nM), demonstrated that the inhibition or obliteration of β2AR abolished the adaptive immunoregulatory effects exerted by E2 during chronic stress. Typically, it is shown that during stress, E2 enhanced PMΦ polarizations into more CD206+ macrophages (anti-inflammatory phenotype) than CD86+ macrophages (proinflammatory phenotype) when β2ARs are not inhibited. However, obliteration of β2AR activities (by its KO or blocker ICI 118,551) obstructs the initially observed estrogenic phenomenon and consequently causes an increase in M1 macrophage phenotype (Figures 5C,D).

DISCUSSION

Unresolved myocardial inflammatory responses have been clinically demonstrated as an underlying factor expediting the pathological remodeling of the heart during stress (Mori et al., 2011; Wilson et al., 2018; Scally et al., 2019). The homeostatic balance between cardiac proinflammatory and anti-inflammatory macrophage phenotypes is crucial for resolving myocardial inflammation and proper heart functioning (Mouton et al., 2018). However, clinical studies have shown that the
myocardia of CSC patients have massive bias infiltrations of proinflammatory macrophages, which prolongs inflammation without timely resolutions to permit reparative functions of anti-inflammatory macrophages. Hence, in post-stress–induced cardiac injuries, the maladaptive proinflammatory responses in the myocardia drives the pathological remodeling of the heart, which is evident by marked fibrosis (Mori et al., 2011; Mouton et al., 2018).
Herein, we demonstrate the mechanistic roles employed by E2 to protect the heart during chronic stress from an immunoregulatory perspective. Morphometric evaluations revealed significant gains in BW resulting from the deficiency of estrogen in the OVX rats under physiological and chronic stress states. This finding provides supporting evidence that E2 is crucial for efficient lipid metabolism. In fact, previous studies have demonstrated that E2 maintains a healthy lipid profile by upregulating bloodstream levels of high-density lipoprotein (HDL) and lowering low-density lipoprotein receptors (LDL) (Lee et al., 2015; Fu et al., 2019; Ndzie Noah et al., 2021). As such, the deficiency of E2 scaffolded disorders in lipid metabolism. In addition, previous work has shown that E2 prevents excessive weight gains, which ultimately impacts positively on cardiac health.

Clinically, demographics clearly show that normally, females have higher heart rates (HR) and cardiac outputs than males of the same age cohort (Wheatley et al., 2014). In menopause, there is a further increase in HR, which results in short-term arrhythmias (heart palpitations) and are attributed to the loss of E2 and possibly β2AR signaling dysregulation (Carpenter et al., 2021). Interestingly, the contrary was found in this study. The obliteration of E2 via ovariectomy resulted in a slight decrease in HR under normal state; however, chronic stress in these OVX rats caused a significant reduction in HR. The possible explanation for this outcome is that inotropy and chronotropic functions of the heart are mediated by β1AR and β2AR; meanwhile, E2 prevents

**FIGURE 4** Estrogen attenuates maladaptive myocardial inflammatory responses chronic stress. (A,B) Representative immunohistochemical staining and graphical presentation of CD68-positive cells (whole macrophages) assessed from the myocardia. (C,D) Representative immunohistochemical staining and graphical presentation of CD86-positive cells (proinflammatory phenotype/M1 macrophages) assessed from the myocardia. (E,F) Representative immunohistochemical staining and graphical presentation of CD206-positive cells (anti-inflammatory phenotype/M2 macrophages) assessed from the myocardia. (G,H) Graphical presentation of M1 macrophage markers, tumor necrosis factor-alpha (TNF-α), and inducible nitric oxide synthase (iNOS) mRNA expressions assessed by RT-qPCR (n = 4 hearts/group). (I,J) Graphical presentation of M2 macrophage markers, transforming growth factor-beta (TGF-β), and arginase 1 (Arg-1) mRNA expressions assessed by RT-qPCR (n = 5 hearts). *p < 0.05 and **p < 0.01; &p < 0.05. Data are presented as mean ± SEM. Data were analyzed using two-way ANOVA and Bonferroni’s multiple comparisons test.
Estrogenic adaptive immunoregulation of macrophage polarization involves modulation of β2-AR signaling activities. (A,B) Representative immunoblots and graphical presentation of β2-AR expressions evaluated from the myocardia. S, Sham; O, OVX; OE, OVX + E2; SI, Sham + ISO; OI, OVX + ISO; OEI, OVX + E2 + ISO (n = 4 hearts/group). (C,D) Representative flow cytometry plots of gated macrophage phenotypes and graphical presentation of their M1 and M2 expression ratios (n ≤ 1 * 10^6 cells). The phenotypic populations of macrophages were quantified using FlowJo. *p < 0.05; **p < 0.001 vs. CD86+ (ISO + E2); ###p < 0.001 vs. CD206+ (ISO + E2 + ICI118551). Data are presented as mean ± SEM. Data were analyzed using two-way ANOVA, followed by Sidak’s post hoc analysis.

Their dysregulations and substantial depletion during stress (Hou et al., 2018; Ndzie Noah et al., 2021). Therefore, E2 deficiency might have permitted dysfunctionalities and downregulation of the β2-ARs during stress, hence the significant decrease in HR. Also, consistent with previous reports, it was found that the cardiac function index; LVSP, LVEDP, +dp/dt, and −dp/dt were unaffected by E2 deficiency under physiological state (Mori et al., 2011; Ribeiro et al., 2013). Even so, chronic stimulation of βARs by ISO during E2 deficiency demonstrated overt cardiac dysfunctions. In contrast, it was demonstrated that E2Endo in the Sham + ISO and OVX + E2 + ISO rats, respectively, ameliorated these heart dysfunctions to sustain cardiac output during stress.

Further investigations sought to characterize the impact of chronic stress on the myocardial structure during E2 deficiency. It was observed that cardiomyocyte diameters generally increased during stress; however, the E2 deficiency permitted maladaptive hypertrophy, which distorted the typical myocardial architecture. This was further proven by the significant upregulations of ANP and BNP in the hearts of OVX rats during chronic stress. Nevertheless, E2Endo (in the Sham rats) showed much potency at minimizing the upregulations of both ANP and BNP during stress, while E2Exo (in the OVX + E2 rats) was unable to downregulate the latter substantially. In conformity with our findings, Goncalves et al. (2018) and others had early demonstrated that E2 exerts antihypertrophic effects via...
GPER (Goldstein et al., 2004). Also, the discrepancies observed between the antihypertrophic effect of E_{2Endo} and E_{2Exo} might have occurred because other ovarian secretions such as vascular endothelial growth factor (VEGF) may complement the efforts of E_{2} in preventing maladaptive cardiomyocyte hypertrophy (Cai et al., 2015). Besides, as suggested by Zhang et al. (2021), unlike the E_{2Exo} treatment dose, which remained constant during CSC modeling, the levels of E_{2Endo} are altered due to the estrous cycle in the Sham and could have also contributed to the observed differences in the antihypertrophic effect of E_{2}. In addition, it was found that obliteration of E_{2} in OVX rats permitted induction of massive interstitial fibrosis; nevertheless, its presence/restoration ameliorated this adverse outcome. We showed that comparatively, E_{2Endo} in the Sham and its supplementation (E_{2Exo}) lessened the extent of fibrosis, just as demonstrated earlier (Mori et al., 2011; Michalson et al., 2018).

The homeostatic balance between proinflammatory and anti-inflammatory macrophages in the myocardia during steady state is crucial for cardiac function, as is the timely trafficking of either of them during injury/cell clearance or reparative process, respectively, essential for preventing adverse heart remodeling (Lavine et al., 2014; Mouton et al., 2018). However, as demonstrated from the postmortem examination of the hearts from CSC patients, proinflammatory macrophages were abundant in the myocardia and were shown to have exacerbated myocardial proinflammatory responses, which may have resulted from stress-induced cardiac insults. The observed biased infiltration of CD86+ macrophages (proinflammatory) hastened the pathological cardiac remodeling as autosied hearts had marked fibrosis (Wilson et al., 2018; Scally et al., 2019). Similar to these clinical findings, it has been shown in rats that stress causes augmentation of myocardial CD86+ macrophage infiltrations, and the phenomena are worsened by E_{2} deficiency (Mori et al., 2011). Following up on these previous studies, consistent findings were made. CD68-positive cell infiltration into the myocardia were increased only under stress conditions; however, E_{2} deficiency augmented their infiltration significantly. Nonetheless, E_{2Endo} and its supplementation (E_{2Exo}) to the rats during stress maximized CD68-positive cell infiltration. Assessing the phenotypic ratios with CD86 and CD206 immunostaining revealed the majority of the CD68-positive cells infiltrating the myocardia when E_{2} is deficient during stress are CD86-positive cells, while CD206-positive cells are less present. Nevertheless, E_{2Endo} and E_{2Exo} reversed these phenomena by enhancing anti-inflammatory responses in the hearts during stress via increasing CD206+ macrophage presence, as similarly reported previously (Xing et al., 2009; Bolego et al., 2013). Validations of the aforementioned findings were done by assessing the mRNA expressions of proinflammatory (TNF-α and iNOS) and anti-inflammatory macrophage (TGF-β and Arg-1) markers from the myocardia of all experimental groups. Similar to the observations of the histological evaluations, TNF-α and iNOS were upregulated during stress and were further elevated significantly when E_{2} is deficient. Also, TGF-β and Arg-1 mRNA expressions were downregulated in the myocardia due to E_{2} deficiency. Conversely, E_{2Endo} exerted anti-inflammatory effects by enhancing TGF-β and Arg-1 while decreasing TNF-α and iNOS mRNA expressions during stress. Although E_{2Exo} upregulated TGF-β and Arg-1 and inhibited TNF-α similarly to E_{2Endo} it was not as potent as E_{2Endo} in downregulating iNOS. The possible explanation of the phenomenon is that ovarian secretions of progesterone might have complimented the inhibitory effects of E_{2Endo}, as it has been reported that besides E_{2}, progesterone decreases iNOS levels in non-cardiac tissue (Menzies et al., 2011). However, progesterone is obliterated in OVX + E_{2} + ISO rats; hence, it might account for iNOS being significantly downregulated in Sham + ISO than OVX + E_{2} + ISO. Nonetheless, the estrogenic anti-inflammatory effects demonstrated here have been similarly reported by Villa et al. (2015) and others (Chen et al., 2021).

Similar to cardiomyocytes, macrophages have profound expressions of β_{2}AR and estrogen receptors (ERs), and the cardioprotective effects conferred by E_{2} have been demonstrated to mostly involved the synergy of ERs and β_{2}AR signaling cascades (Kang et al., 2012; Hou et al., 2018; Machuki et al., 2019; Ndzie Noah et al., 2021). Hence, to elucidate the immunoregulatory mechanisms employed by E_{2} to facilitate more CD206+ macrophage polarizations to timely resolve inflammation during stress, we investigated the possible involvement of β_{2}AR signaling modulation by E_{2}-induced cascades. In conformity with our initial speculations, β_{2}AR expressions from apical myocardia (constituting cardiomyocytes and infiltrated macrophages) were found to be significantly depleted during chronic stress due to E_{2} deficiency, as the presence of E_{2Endo} in the Sham and the supplementation of E_{2Exo} in OVX rats showed a minimal reduction in the expression of the receptor under the same stress condition. Further investigations of β_{2}AR involvement deployed the isolations of PMs from female WT and β_{2}AR-KO mice as well as the use of β_{2}AR blocker ICI 118,551 to ascertain if E_{2} induced any variations in the phenotypic ratios of the macrophages during stress was affected by impeding β_{2}AR signaling. We report that the estrogenic signaling facilitates adaptive immunoregulation by ensuring CD206+ macrophage polarizations to timely resolve inflammation as reported by others (Keselman et al., 2017). However, for the first time, we show the underlying mechanism involves interplays of E_{2}, ERs and β_{2}AR signaling during stress. Flow cytometric evaluations show that E_{2} treatments during stress increased CD206+ macrophage polarizations against CD86+ macrophages; however, the deletion/inhibition of β_{2}AR impaired this phenomenon. These observations are possibly because the bioavailability of nitric oxide (NO), which is produced via β_{2}AR-G_{αι}-PI3K-Akt-mediated signaling cascade, is crucial for the polarization of macrophages from proinflammatory to the anti-inflammatory phenotype (De Nigris and Prattichizzo, 2021). As such, blockade of β_{2}AR signaling disrupts NO bioavailability and abolishes this adaptive immunoregulatory mechanism. Also, E_{2} had been shown previously to exert these anti-inflammatory effects primarily via estrogen receptor alpha (ERα) (Bolego et al., 2013; Campbell et al., 2014), and although we have demonstrated here the essential involvement of β_{2}AR to facilitate the polarizations of CD206+ macrophages, there are
apparent interplays among E2, ERs, and β2AR to ensure this immunomodulation during stress. Intriguingly, E2 and ER activities downregulate GRK2, which otherwise would have induced the homologous desensitization and downregulation of β2AR during stress (Abraham et al., 2018; Arcones et al., 2021). Therefore, E2 and ERs indirectly sustain the bioavailability of NO via β2AR-Gαs-PI3K-Akt signaling by preventing dysregulation of the receptor during stress and enhancing the β2AR-mediated CD206+ macrophage polarization.

Taken together, the findings from this study demonstrate the immunoregulatory mechanisms employed by E2 to confer cardioprotection and lower the incidence of CSC in premenopausal women as compared with postmenopausal women and males of all age cohorts. E2 exerts this immunoregulatory myocardia protection to prevent pathological cardiac remodeling during stress by ensuring the timely resolution of myocardial proinflammatory responses and enhancing reparative functions of CD206+ macrophage. More importantly, we demonstrate here that the adaptive modulation of macrophage phenotypes by E2 during stress requires the mediation of β2AR signaling. The classical interplays among E2, ERs, and β2AR discussed by Ndzie Noah et al. (2021) are also shown here, as E2 and ER activities are in turn required to prevent β2AR dysregulations and dysfunctionalities during stress. From a therapeutic standpoint, the findings from this study reechoes the essence of E2 replacement therapy (E2RT) in postmenopausal women, as it reduces the incidence of CSC. However, it is recommended that E2RT is initiated within 5−6 years after menopause so as to explore its therapeutic benefits fully while circumventing the adverse outcomes reported by the Women’s Health Initiative from their randomized controlled trial (Rossouw et al., 2002; Michalson et al., 2018; Ndzie Noah et al., 2021). Finally, it is deemed necessary to point out the limitations of this study due to its clinical significance. β1ARs are essential for myocardial functions and might play other immunologic roles facilitated by E2, but they have not been elucidated previously nor in this study. Also, in some instances (Figures 3E,F), (Figures 4C−J), it is shown that E2Exo did not confer anti-inflammatory effects as E2Endo did. However, the fact that other ovarian secretions such as progesterone can complement the anti-inflammatory effects of E2Endo, but are obliterated by ovariectomy in the E2Exo treatment group might explain the observed differences. The estrous cycle in the Shams causing alterations in E2Endo levels while E2Exo treatment dosage used remained constant might also account for the shown slight variations in E2Endo and E2Exo effects. Therefore, we stand with Zhang et al. (2021) in suggesting that E2RT should be given at dosages that mimic the concentrations of the estrous cycle to eliminate the observed variations in its cardioprotection efficacy. This will enhance the exploitation of the therapeutic potentials of E2RT in attenuation/prevention of CSC via immunomodulation in postmenopausal women.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the Experimental Animal Centre of Xuzhou Medical University and the Animal Ethics Committee of Xuzhou Medical University (permit no: xz11-12540).

**AUTHOR CONTRIBUTIONS**

HH conceived the experiment idea. HS, HH, GKA, and QW designed the experiments. HH and GKA isolated and cultured PMs. HH, TM, and YM made animal models. HH, JG, MS, and LF performed cardiac function and histological assessments. HH, GKA, QW, and HS analyzed and interpreted the results. Based on the contributions of all authors, HH drafted the initial manuscript and GKA revised it entirely. HH, GKA, QW, TM, YM, JG, MS, LF, RR, and ZG proofread and approved the manuscript in its current form.

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**REFERENCES**

Abraham, A. D., Schattauer, S. S., and Reichard, K. L. (2018). Estrogen regulation of GRK2 inactivates kappa opioid receptor signaling mediating analgesia, but not aversion. J. Neurosci. 38, 8031−8043. doi: 10.1523/JNEUROSCI.0653-18.2018

Adzika, G. K., Machuki, J. O., Shang, W., Hou, H., Ma, T., Wu, L., et al. (2019). Pathological cardiac hypertrophy: the synergy of adenylyl cyclases inhibition in cardiac and immune cells during chronic catecholamine stress. J. Mol. Med. 97, 897−907. doi: 10.1007/s00109-019-01790-0

Arcones, A. C., Martinez-Cignoni, M. R., Vila-Bedmar, R., Yáñez, C., and Lladó, I. (2021). Cardiac GRK2 protein levels show sexual dimorphism during aging and are regulated by ovarian hormones. Cells 10:673. doi: 10.3390/cells10030673

Boese, A. C., Kim, S. C., Yin, K. J., Lee, J. P., and Hamblin, M. H. (2017). Sex differences in vascular physiology and pathophysiology: estrogen and androgen signaling in health and disease. Am. J. Physiol. Heart Circ. Physiol. 313, H524−H545. doi: 10.1152/ajpheart.00217.2016

Bolego, C., Cignarella, A., Staels, B., and Chinetti-Gbaguidi, G. (2013). Macrophage function and polarization in cardiovascular disease: a role of estrogen signaling? J. Neurosci. 38, 8031−8043. doi: 10.1523/JNEUROSCI.0653-18.2018

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**REFERENCES**

Abraham, A. D., Schattauer, S. S., and Reichard, K. L. (2018). Estrogen regulation of GRK2 inactivates kappa opioid receptor signaling mediating analgesia, but not aversion. J. Neurosci. 38, 8031−8043. doi: 10.1523/JNEUROSCI.0653-18.2018

Adzika, G. K., Machuki, J. O., Shang, W., Hou, H., Ma, T., Wu, L., et al. (2019). Pathological cardiac hypertrophy: the synergy of adenylyl cyclases inhibition in cardiac and immune cells during chronic catecholamine stress. J. Mol. Med. 97, 897−907. doi: 10.1007/s00109-019-01790-0

Arcones, A. C., Martinez-Cignoni, M. R., Vila-Bedmar, R., Yáñez, C., and Lladó, I. (2021). Cardiac GRK2 protein levels show sexual dimorphism during aging and are regulated by ovarian hormones. Cells 10:673. doi: 10.3390/cells10030673

Boese, A. C., Kim, S. C., Yin, K. J., Lee, J. P., and Hamblin, M. H. (2017). Sex differences in vascular physiology and pathophysiology: estrogen and androgen signaling in health and disease. Am. J. Physiol. Heart Circ. Physiol. 313, H524−H545. doi: 10.1152/ajpheart.00217.2016

Bolego, C., Cignarella, A., Staels, B., and Chinetti-Gbaguidi, G. (2013). Macrophage function and polarization in cardiovascular disease: a role of estrogen signaling?
Hou et al. (2021). Catalpol enhances estradiol's cardioprotection in ovariectomized rats. J. Endocrinol. 212, 61–69. doi: 10.1530/JOE-11-0811

Machuki, O. Z., Zhang, H. Y., Geng, J., Fu, L., Adzika, G. K., Wu, L., et al. (2019). Estrogen regulation of cardiac AMPK-L type Ca(2+) channel pathway modulates sex differences in basal contraction and responses to β2AR-mediated stress in left ventricular apical myocytes. Cell Commun. Signal. 17:34. doi: 10.1186/s12964-019-0345-2

Medeiros, K., O’Connor, M. J., Baicu, C. F., Fitzgibbons, T. P., Shaw, P., Tighé, D. A., et al. (2014). Systolic and diastolic mechanics in stress cardiomyopathy. Circulation 129, 1659–1667. doi: 10.1161/CIRCULATIONAHA.113.002781

Menzies, F. M., Henríquez, F. L., Alexander, J., and Roberts, C. W. (2011). Selective inhibition and augmentation of alternative macrophage activation by progesterone. Immunology 134, 281–291. doi: 10.1111/j.1365-2567.2011.03488.x

Michaolson, K. T., Groban, L., Howard, T. D., Shively, C. A., Sophonsritsuk, A., Appt, S. E., et al. (2018). Estradiol treatment initiated early after ovarioectomy regulates myocardial gene expression and inhibits diastolic dysfunction in female cynomolgus monkeys: potential roles for calcium homeostasis and extracellular matrix remodeling. J. Am. Heart Assoc. 7:e009769. doi: 10.1161/ JAHA.118.009769

Mori, T., Kai, H., Kajimoto, H., Koga, M., Kudo, H., Takayama, N., et al. (2011). Enhanced cardiac inflammation and fibrosis in ovariectomized hypertensive rats: a possible mechanism of diastolic dysfunction in postmenopausal women. Hypertens. Res. 34, 496–502. doi: 10.1038/htr.2010.261

Mouton, A. J., DeLeon-Pennell, K. Y., Rivera Gonzalez, O. J., Flynn, E. R., Freeman, T. C., Saucerman, J. I., et al. (2018). Mapping macrophage polarization over the myocardial infarction time continuum. Basic Res. Cardiol. 113:26. doi: 10.1007/s00395-018-0668-x

Ndzie Noah, M. L., Adzika, G. K., Mprah, R., Adekunle, A. O., Adum-Amanwaa, J., and Sun, H. (2021). Sex–gender disparities in cardiovascular diseases: the effects of estrogen on eNOS, lipid profile, and NFATs during catecholamine stress. Front. Cardiovasc. Med. 8:33946. doi: 10.3389/fcvm.2021.63946

Paur, H., Wright, P. T., Sikkil, M. B., Tranter, M. H., Mansfield, C., O’Gara, P., et al. (2012). High levels of circulating epinephrine trigger apical cardiodepression in a β2 adrenergic receptor/Gi-dependent manner: a new model of Takotsubo cardiomyopathy. Circulation 126, 697–706. doi: 10.1161/CIRCULATIONAHA.111.115911

Ray, A., and Dittel, B. N. (2010). Isolation of mouse peritoneal cavity cells. J. Vis. Exp. 35:1488. doi: 10.3791/1488

Ren, J., Hintz, K. R., Rough, Z. K., Duan, J., Colligan, P. B., Ren, B. H., et al. (2003). Impact of estrogen replacement on ventricular myocyte contractile function and protein kinase β/Akt activation. Am. J. Physiol. Heart Circ. Physiol. 284, H1800–H1807. doi: 10.1152/ajpheart.00866.2002

Ribiero, F. R., Potratz, F. F., Pawan, B. M., Forechi, L., Lima, F. M., Fiorim, J., et al. (2013). Cardiiodil prevents ovarioectomy-induced myocardial contractile dysfunction in female rat. PLoS One 8:e53226. doi: 10.1371/journal.pone.0053226

Rossouw, J. E., Anderson, G. L., Prentice, R. L., LaCroix, A. Z., Koopberg, C., Stefanick, M. L., et al. (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women’s Health initiative randomized controlled trial. JAMA 288, 321–333. doi: 10.1001/jama.288.3.321

Scally, C., Abbas, H., Ahearn, T., Srinivasan, I., Mezincescu, A., Rudd, A., et al. (2019). Myocardial and systemic inflammation in acute stress-induced (Takotsubo) cardiomyopathy. Circulation 139, 1581–1592. doi: 10.1161/ CIRCULATIONAHA.118.037975

Villa, A., Rizzi, N., Vegato, E., Ciana, P., and Maggi, A. (2015). Estrogen accelerates the resolution of inflammation in macrophagic cells. Sci. Rep. 5:15224. doi: 10.1038/srep15224

Wheatley, C. M., Snyder, E. M., Johnson, B. D., and Olson, T. P. (2014). Sex differences in cardiovascular function during submaximal exercise in humans. Springerplus 3:445. doi: 10.1186/2193-1801-3-445

Wilson, H. M., Cheyne, L., Brown, P. A. J., Kerr, K., Hannah, A., Srinivasan, J., et al. (2018). Characterization of the myocardial inflammatory response in acute stress-induced (Takotsubo) cardiomyopathy. JACC Basic Transl. Sci. 3, 766–776. doi: 10.1016/j.jatbs.2018.08.006
Xing, D., Nozell, S., Chen, Y. F., Hage, F., and Oparil, S. (2009). Estrogen and mechanisms of vascular protection. *Arterioscler. Thromb. Vasc. Biol.* 29, 289–295. doi: 10.1161/ATVBAHA.108.182279

Youssef, M. E., El-Mas, M. M., Abdelrazek, H. M., and El-Azab, M. F. (2021). α7-nAChR-mediated therapeutic angiogenesis accounts for the advantageous effect of low nicotine doses against myocardial infarction in rats. *Eur. J. Pharmacol.* 898:173996. doi: 10.1016/j.ejphar.2021.173996

Zhang, L., Li, C., Yang, L., Adzika, G. K., Machuki, J. O. A., Shi, M., et al. (2021). Estrogen protects vasomotor functions in rats during catecholamine stress. *Front. Cardiovasc. Med.* 8:679240. doi: 10.3389/fcvm.2021.679240

Zhou, R., Ma, P., Xiong, A., Xu, Y., Wang, Y., and Xu, Q. (2017). Protective effects of low-dose rosuvastatin on isoproterenol-induced chronic heart failure in rats by regulation of DDAH-ADMA-NO pathway. *Cardiovasc. Ther.* 35:e12241. doi: 10.1111/1755-5922.12241

Zhu, Y., Zhang, L., Lu, Q., Gao, Y., Cai, Y., Sui, A., et al. (2017). Identification of different macrophage subpopulations with distinct activities in a mouse model of oxygen-induced retinopathy. *Int. J. Mol. Med.* 40, 281–292. doi: 10.3892/ijmm.2017.3022

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