Effects of 17beta-estradiol on cardiac apoptosis in ovariectomized rats

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Objectives   Cardiac apoptosis was found in ovariectomized rats without ischemia. Limited information regarding the protective effects of 17β-estradiol (E2) on cardiac Fas-dependent and mitochondria-dependent apoptotic pathways after post-menopause or bilateral oophorectomy in women was available.
Methods   Forty-eight female Wistar rats at 6-7 months of age were divided into sham-operated group (Sham, n = 16) and bilateral ovariectomized group (n = 32). After 4 weeks of operation, rats in ovariectomized group were injected intraperitoneally with either saline (OVX, n = 16) or 10 µg/kg/day 17β-estradiol (E2) for 10 weeks (OVX-E2, n = 16). The excised hearts were measured by Hematoxylin-eosin staining, DAPI staining, positive TUNEL assays, and Western Blotting.
Results   17β-estradiol (E2) decreased OVX-induced cardiac widely dispersed TUNEL-positive apoptotic cells. 17β-estradiol (E2) decreased OVX-induced TNF-alpha, Fas ligand (Fas L), Fas death receptors (Fas), Fas-associated death domain (FADD), activated caspase 8, and activated caspase 3 (Fas pathways). 17β-estradiol (E2) decreased OVX-induced proapoptotic t-Bid, Bax, Bax-to-Bcl2 ratio, Bax-to-BclXL ratio, activated caspase 9, and activated caspase 3 as well as increased anti-apoptotic Bcl2 and Bcl-XL relative to OVX (mitochondria pathway).
Conclusions   Our findings suggest that chronic 17β-estradiol (E2) treatment can prevent ovariectomy-induced cardiac Fas-dependent and mitochondria-dependent apoptotic pathways in rat models. The findings may provide one of possible mechenisms of 17β-estradiol (E2) for potentially preventing cardiac apoptosis after bilateral ovariectomy or menopause. Copyright © 2010 John Wiley & Sons, Ltd.

KEY WORDS — heart; apoptosis; menopause; ovariectomy; oophorectomy; caspase; estrogen; estradiol

INTRODUCTION

Menopause and early ovariectomy (oophorectomy) play a pathological role in developing coronary diseases and deteriorating cardiovascular conditions [1–5]. About 55% of female deaths in Europe were caused by cardiovascular diseases, such as myocardial infarction and heart failure [6]. A report in 2006 from 38283 women showed that each 5-year increment in age after post-menopause was associated with a 44% increase of the risk of heart failure and with a 52% risk of all-cause mortality [7]. The cellular apoptosis in cardiomyocytes is one of very critical pathological mechanisms to cause heart failure [8]. Most of previous studies regarding cardiovascular diseases after menopause or estrogen deficiency focus on the investigation of coronary artery diseases [1–5], but very limited information regarding cardiac apoptosis after menopause is available.

Apoptosis, a physiological program of cellular death, in heart may contribute to many cardiac disorders [9–10]. Cardiac apoptosis was found by our laboratory in many chronic cardiometablic or stressful conditions such as obesity [11–12], hypertension [10,13], diabetes [14], ovariectomy [15], long-term

hypoxia [16–17], and smoking exposure [18]. The occurrence of apoptosis is recognized as a predictor of adverse outcomes in patients with cardiac diseases or heart failure [19]. The 'extrinsic' Fas receptor-dependent (type I) apoptotic pathway and 'intrinsic' mitochondria-dependent (type II) apoptotic pathway were believed to be two of the major pathways directly to trigger cardiac apoptosis [9,20]. The type I apoptotic pathway was initiated by binding of Fas ligand to the Fas receptor, which results in clustering of receptors and initiates the extrinsic pathway [20]. Fas receptor oligomerization recruits FADD and pro-caspase 8 to the complex and results in the activation of caspase 8. The activated caspase 8 cleaves pro-caspase 3, which then undergoes autocatalysis to form active caspase 3, a principle effector caspase of apoptosis [20–21]. The 'intrinsic' mitochondria-dependent (type II) apoptotic pathway starts from within the cell resulting in the release of a number of pro-apoptotic factors from the intermembrane space of mitochondria [20,22]. The mitochondria is the main site of action for members of the apoptosis-regulating protein family exemplified by Bcl-2 family, such as Bcl-2 and Bax [23–24]. Pro-apoptotic and anti-apoptotic Bcl-2 family members can homodimerize or heterodimerize to each other, and appear to interact with and neutralize each other, so that the relative balance of these effectors strongly influences cytochrome *c* release and cell fate [23–25]. Bcl-2 and Bcl-XL, anti-apoptotic proteins, prevents cytochrome *c* release whereas t-Bid and Bax, pro-apoptotic proteins, enhance cytochrome *c* release from mitochondria [20,23–26]. When cytochrome *c* is released from mitochondria into cytosol, it is responsible for activating caspase-9, which further activates caspase-3 and executes the apoptotic program [20,24]. In our previous study, the key components of Fas-dependent apoptosis (Fas ligand, Fas death receptors, Fas-associated death domain (FADD), activated caspase 8, and activated caspase 3) and key components of mitochondria-dependent apoptosis (Bax, Bax-to-Bcl2 ratio, cytosolic cytochrome *c*, activated caspase 9, and activated caspase 3) were significantly increased in ovariectomized rats hearts with widely dispersed apoptosis [15]. However, the therapeutic effects of ovariectomy-induced cardiac apoptosis have not been investigated.

17β-estradiol (estradiol), the most abundant ovarian estrogen, affords cardiovascular protection in coronary diseases [27–28]. However, the underlying mechanism of 17β-estradiol on heart failure or cardiac apoptosis in post-menopausal or early ovariectomized women was still unclear. In the current study, we hypothesized that chronic 17β-estradiol (E2) treatment may prevent cardiac Fas-dependent and/or mitochondria-dependent pathways in ovariectomized rats with cardiac apoptosis.

MATERIALS AND METHODS

Animals

Forty-eight female Wister rats were purchased from National Laboratory Animal Center, Taiwan. Ambient temperature was maintained at 25°C and the animals were kept on an artificial 12-h light-dark cycle. The light period began at 7:00 A.M. Rats were provided with standard laboratory chow (Lab Diet 5001; PMI Nutrition International Inc., Brentwood, MO, USA) and water *ad libitum*. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan.

Ovariectomy and Sham operation

All forty-eight rats were divided into two groups, i.e. sham-operated group (Sham, n = 16) and bilateral ovariectomized group (n = 32). All animals in bilateral ovariectomized group were conducted by survival surgical procedures with aseptic technique at age of 5-6 months. After anesthetized with intramuscular injection of ketamine (100 mg/kg), the lumbar dorsum was shaved bilaterally and the exposed skin was cleaned with a 75% alcohol wipe followed by a 10% povidone-iodine scrub. For each ovary, a 2 cm dorsal flank incision penetrating the abdominal cavity was made. After the par ovarian fatty tissues were identified and retracted, the ovarian arteries were ligated and the bilateral ovaries were removed. The wound was then closed using 4-O sterile suture and each rat was injected with Penicillin-G procaine (0.2 ml, 20,000 IU, IM). The sham-operated group (sham) underwent the same surgical procedure except for the ligation of ovarian arteries and removal of the ovaries. After bilateral ovariectomy or sham operation, the rats were kept individually in plastic cages for recovery for about 10 days, and then grouped back to their home cages.

Chronic 17β-estradiol (E2) treatment

17β-estradiol (E2) was purchased from Sigma Chemical Company (St. Louis, MO, USA). After 4 weeks of operation, rats in bilateral ovariectomized group were divided into two subgroups, each subgroup of which was injected intraperitoneally with either saline (OVX group, n = 16) for 10 weeks, or 10 μg/kg/day 17β-estradiol (E2) for 10 weeks (OVX-E2 group, n = 16).

Cardiac characteristics

All forty-eight rats in three groups (i.e Sham, OVX, and OVX-E2) were weighed and decapitated at age of about 8-9 months. The 8 hearts of rats in each group were soaked in formalin and further analyzed by Hematoxylin-eosin, DAPI staining and TUNEL assay. The other 8 hearts of rats in each group were excised, cleaned, frozen, and further analyzed by Western Blotting. The right tibias were also separated and tibia length was measured by the electronic digital vernier caliper for correcting the whole heart weight. The ratios of the whole heart weight (WHW) to body weight (BW), the ratios of the left ventricular weight (LVW) to body weight (BW), the left ventricle weight (LVW) to the whole heart

weight (WHW), the whole heart weight to tibia length, and the left ventricle weight to tibia length were calculated.

Hematoxylin-eosin staining

The hearts from animals were excised and were soaked in formalin and covered with wax. Slides were prepared by deparaffinization. They were passed through a series of graded alcohols (100%, 95% and 75%), 15 minutes for each. The slides were then dyed with hematoxylin and eosin. After gently rinsing with water, each slide was then soaked with 85% alcohol, 100% alcohol I and II for 15 minutes each. At the end, they were soaked with Xylene twice. Photomicrographs were obtained using Zeiss Axiophot microscopes.

TISSUE EXTRACTION

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples in a lysis buffer (20mM Tris, 2mM EDTA, 50mM 2-mercaptoethanol, 10% glycerol, PH 7.4, protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail (sigma)) at a ratio of 100 mg tissue/1ml buffer for 1 min. The homogenates were placed on ice for 10 min and then centrifuged at $12,000\,g$ for 40 min twice. The supernatant was collected and stored at -70°C for further experiments.

Electrophoresis and Western Blot

Protein concentration of cardiac tissue extracts was determined by the Bradford method (Bio-Rad Protein Assay, Hercules, CA). Protein samples ($50\,\mu g$/lane) were separated on a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a constant voltage of 75 V. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, $0.45\,\mu m$ pore size) with a transferring apparatus (Bio-rad). PVDF membranes were incubated in 5% milk in TBS buffer. Primary antibodies including TNF-alpha, Fas ligand, Fas receptor, FADD, t-Bid, Bcl-XL, Bax, caspase 8, caspase-9, caspase-3 and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Bcl-2 (BD) were diluted to 1:500 in antibody binding buffer overnight at 4°C. The immunoblots were washed three times in TBS buffer for 10 min and then immersed in the second antibody solution containing goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, or donkey anti goat IgG-HRP (Santa Cruz) for 1 hour and diluted 500-fold in TBS buffer. The immunoblots were then washed in TBS buffer for 10 min three times. The immunoblotted proteins were visualized by using an enhanced chemiluminescence ECL western Blotting luminal Reagent (Santa Cruz, CA, USA) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan). Densitometric analysis of immunoblots was performed by AlphaImager 2200 digital imaging system (Digital Imaging System, San Leandro, CA, USA).

Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay and 4'-6-Diamidino-2-phenylindole (DAPI) staining

After the hearts were excised, the hearts were soaked in formalin, dehydrated through graded alcohols, and embedded in paraffin wax. In heart tissues, the 0.2-μm thick paraffin sections were cut from paraffin-embedded tissue blocks. The tissue sections were deparaffinized by immersing in xylene, and rehydrated. Next, the sections were incubated with proteinase K, washed in phosphate-buffered saline, incubated with permeabilisation solution, blocking buffer, and then washed twice with PBS. The terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP for 60 min at 37 °C using an apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA) was used for detection. After washing PBS twice, the sections were also stained with 4',6-diamidine-2-phenylindole dihydrochloride (DAPI, Sigma) for 5 min to detect cell nucleus by UV light microscopic observations (blue). TUNEL-positive nuclei (fragmented DNA) fluoresced bright green at 450-500 nm, whereas DAPI-positive nuclei (intact DNA) fluoresced blue at 360 nm. The mean number of TUNEL-positive and DAPI-labeled cells was counted for at least 5-6 separate fields x 2 slices x 3 left ventricle regions (upper, middle, lower) excised from six rat hearts in each group. All counts were performed by at least 2 independent individuals in a blinded manner.

Statistical Analysis

The data of whole heart weight index, protein levels, and the percentage of TUNEL positive cells relative to total cells assay were compared among groups of animals in Sham, OVX, and OVX-E2 groups using one-way analysis of variance (ANOVA) with pre-planned contrast comparison. The ANOVA was analyzed by SPSS statistical software (version 10.0) and was based on a general linear model (GLM). Sham group serves as negative control group for other groups and OVX serves as non-therapeutic group for therapeutic groups. In all cases, a difference at $P < 0.05$ was considered statistically significant.

RESULTS

Body weight and cardiac characteristics

Body weight, whole heart weight, left ventricular weight in OVX groups were more than Sham animals whereas those were less in OVX-E2 group than OVX group (Table 1). However, the whole heart weight corrected by body weight and the left ventricle weight corrected by body weight were similar among these three groups. The ratio of whole heart weight to tibia length and the ratio of left ventricular weight to tibia length were significantly higher in OVX group than those in Sham group, but were lower in OVX-E2 than those in OVX group (Table 1).

Table 1. Cardiac characteristics of sham, ovariectomized group and ovariectomy with 17β-estradiol (E2) treatment

	Sham	OVX	OVX-E2
Number of animals	8	8	8
Body weight (g)	291 ± 14	349 ± 25**	306 ± 21#
Whole heart weight (g)	0.77 ± 0.08	0.91 ± 0.07*	0.77 ± 0.08#
Left ventricle weight (g)	0.55 ± 0.05	0.65 ± 0.04**	0.56 ± 0.04#
Whole heart weight (g) / Body weight (g)*10^3	2.64 ± 0.30	2.62 ± 0.08	2.52 ± 0.08
Left ventricle weight (g) / Body weight (g)*10^3	1.89 ± 0.11	1.87 ± 0.09	1.83 ± 0.10
Left ventricle weight (g) / Whole heart weight	0.72 ± 0.05	0.71 ± 0.03	0.73 ± 0.02
Whole heart weight (g)/ Tibia length (mm)*10^3	19.79 ± 0.03	23.32 ± 0.03*	19.69 ± 0.02#
Left ventricle weight (g) / Tibia length (mm)*10^3	14.14 ± 0.02	16.67 ± 0.02**	14.32 ± 0.03#

Values are means ± SD. Sham, sham-operated (Sham) rats; OVX, Ovariectomized rats; OVX-E2, Ovariectomized rats with 17β-estradiol (E2, 10 μg/kg/day, IP for 10 weeks); *$P < 0.05$, ** $P < 0.01$ Significant differences from control sham group. #$P < 0.05$, Significant differences between Ovx group and Ovx with 17β-estradiol (E2) treatment group.

Cardiac histopathological TUNEL-postive changes

To understand the myocardial architecture and cardiac apoptotic activity in OVX treated with 17β-estradiol (E2), we did a histopathological analysis of left ventricular tissue with Hematoxylin-eosin staining and apoptosis-positive cardiac cells in Sham, OVX, and OVX-E2 groups. Hearts stained with hematoxylin-eosin showed that the ventricular myocardium in the Sham group showed normal architecture with normal interstitial space. In contrast, based on subjective interpretation of the overall histopathological appearance, the abnormal myocardial architecture was observed in OVX, which became normalized in OVX-E2 (Figure 1A). TUNEL assay showed that TUNEL-positive cardiac cells increased in the OVX group and become significantly less in OVX-E2 (Figure 1).

Upstream components of cardiac Fas receptor dependent apoptotic pathways

To investigate the upstream components of cardiac Fas-dependent apoptotic signaling pathways in OVX treated with 17β-estradiol (E2), the protein levels of TNF-α, Fas ligand, Fas receptor and FADD in the excised hearts of Sham, OVX, and OVX-E2 groups were examined by Western blotting. Compared with the Sham group, the levels of TNF-α, Fas ligand (Figure 2), Fas receptor, and FADD (Figure 3) were significantly higher in the OVX group. In addition, the levels of OVX-induced TNF-α, Fas ligand (Figure 2), Fas receptor, and FADD (Figure 3) were significantly ($P < 0.01$) lower in OVX-E2 groups, compared with those in OVX.

Upstream components of cardiac mitochondria-dependent apoptotic pathways

To investigate the mediator that connects the Fas death receptor to the mitochondrial apoptosis pathway in the OVX, we examined the protein levels of t-Bid by Western Blotting. The protein level of t-Bid was significantly were significantly higher in OVX group than those in Sham group. The protein level of t-Bid became less in OVX-E2 than that in OVX (Figure 4). To further understand the cardiac Bcl-2

family in mitochondria-dependent apoptotic pathways in OVX treated with 17β-estradiol (E2), we examined the protein levels of the Bcl-2 family (Bcl-2, Bcl-XL, Bax) in the excised hearts of Sham, OVX, and OVX-E2 groups by
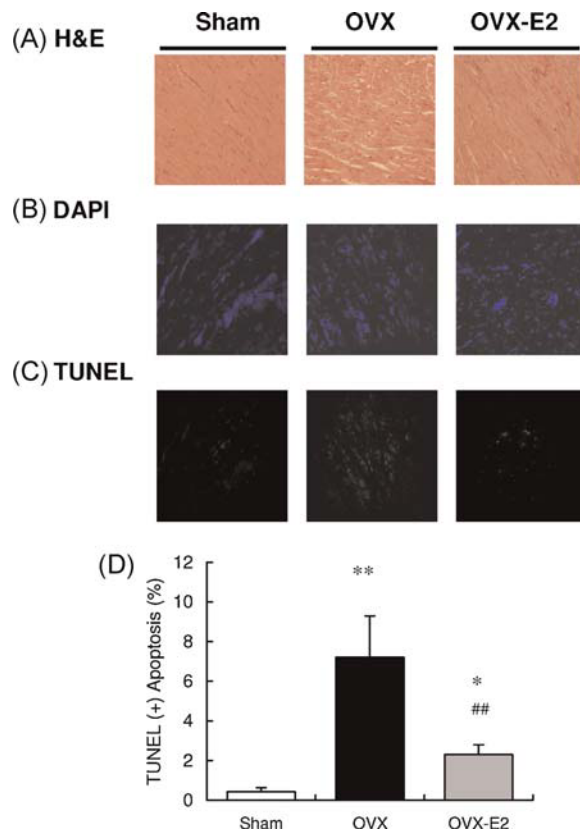

Figure 1. Representative analysis of cardiac tissue sections with (A) Hematoxylin and eosin staining (H&E), (B) 4',6-diamidino-2-phenylindole (DAPI) staining, and (C) Terminal deoxynucleotidyltransferase UTP Nick End Labeling (TUNEL) assay in sham-operated rats (Sham), ovariectomized rats (OVX), and ovariectomized rats with 17β-estradiol (E2, 10 μg/kg/day, IP for 10 weeks) (Ovx-E2). The images were magnified by 400 times. (D) Bars present the percentage of TUNEL positive cells relative to total cells (6 rats x 30 scope field count in each group). *$P < 0.05$, **$P < 0.01$, significant differences from Sham group. ##$P < 0.01$, significant differences from OVX group. This figure is available in colour online at www.interscience.wiley.com/journal/cbf
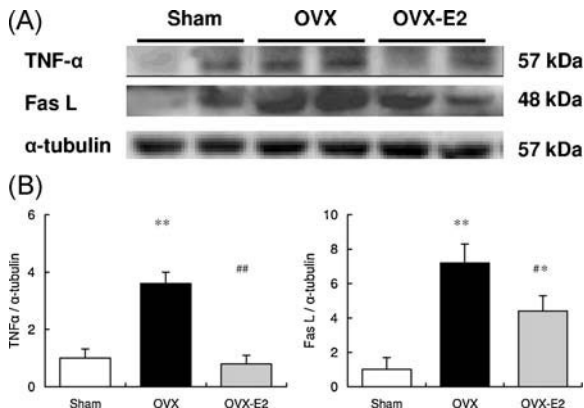
Figure 2. (A)The representive protein products of TNF-α and Fas ligand (Fas L) extracted from the left ventricles of excised hearts in sham-operated rats (Sham), ovariectomized rats (OVX), and ovariectomized rats with 17β-estradiol (E2, 10 μg/kg/day, IP for 10 weeks) (OVX-E2) were measured by Western Blotting analysis. (B) Bars represent the relative protein quantification on the basis of α-tubulin, and indicate mean values ± SD (n = 6 in each group). $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences from Sham group. $^{#}P < 0.05$, $^{##}P < 0.01$, significant differences from OVX group.

Western Blotting. Pro-apoptotic proteins of Bax, Bax-to-BclXL ratio and Bax-to-Bcl2 ratio were significantly higher in OVX group than those in Sham group. Bax, Bax-to-BclXL ratio and Bax-to-Bcl2 ratio become less in OVX-E2 than those in OVX. On the other hand, anti-apoptotic Bcl-XL (not Bcl2) proteins were significantly lower in OVX group than Sham group, but in contrast anti-apoptotic Bcl-XL and Bcl2 proteins were significantly higher in OVX-E2 group than OVX group (Figure 4).
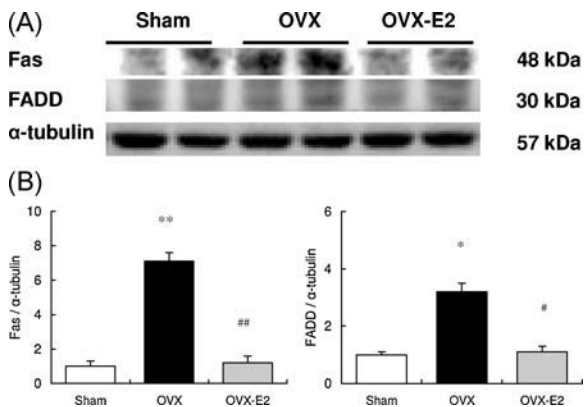

Figure 3. (A)The representive protein products of Fas receptor (Fas) and Fas-associated death domain (FADD) extracted from the left ventricles of excised hearts in sham-operated rats (Sham), ovariectomized rats (OVX), and ovariectomized rats with 17β-estradiol (E2, 10 μg/kg/day, IP for 10 weeks) (OVX-E2) were measured by Western Blotting analysis. (B) Bars represent the relative protein quantification on the basis of α-tubulin, and indicate mean values ± SD (n = 6 in each group). $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences from Sham group. $^{#}P < 0.05$, $^{##}P < 0.01$, significant differences from OVX group.
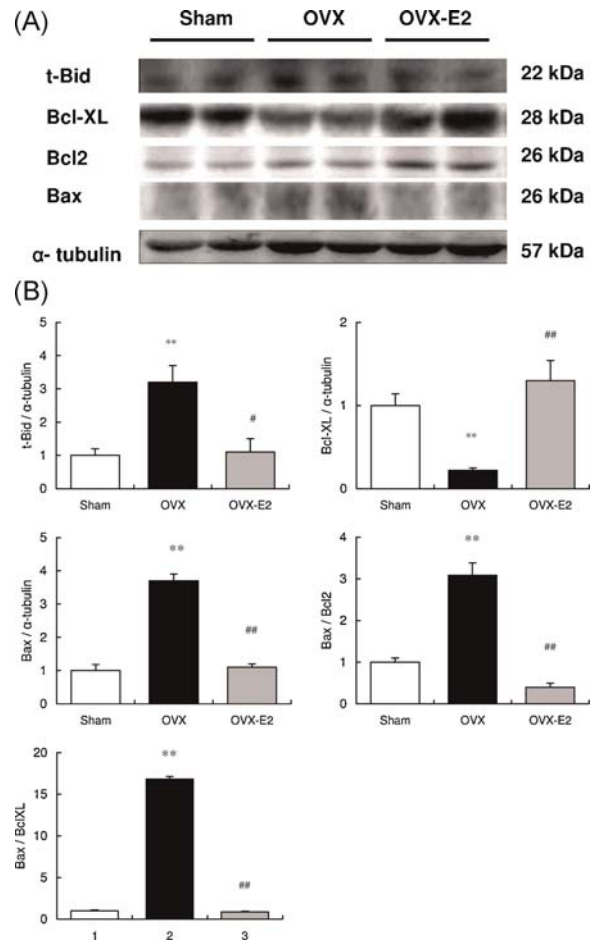

Figure 4. (A)The representive protein products of t-Bid, BclXL, Bcl2, and Bax extracted from the left ventricles of excised hearts in sham-operated rats (Sham), ovariectomized rats (OVX), and ovariectomized rats with 17β-estradiol (E2, 10 μg/kg/day, IP for 10 weeks) (OVX-E2) were measured by Western Blotting analysis. (B) Bars represent the relative protein quantification on the basis of α-tubulin or the ratios of Bax to BclXL and Bax to Bcl2 and indicate mean values ± SD (n = 6 in each group). $^{**}P < 0.01$, significant differences from Sham group. $^{#}P < 0.05$, $^{##}P < 0.01$, significant differences from OVX group.

Downstream components of cardiac Fas-dependent and mitochondria-dependent apoptotic pathways

To identify the downstream components of cardiac Fas (caspase 8 and 3) and mitochondria (caspase 9 and 3) dependent apoptotic pathways, the protein levels of activated caspase 8, 9 and 3 were measured in the excised hearts of Sham, OVX, and OVX-E2 groups by Western Blotting. The protein levels of activated caspase 8 (Fas), caspase 9 (mitochondria), and caspase 3 (mitochondria & Fas) protein products were higher in the OVX group than Sham group. The activated caspase 8, caspase 9, and caspase 3 protein products in the OVX-E2 group become much lower than those in OVX (Figure 5).
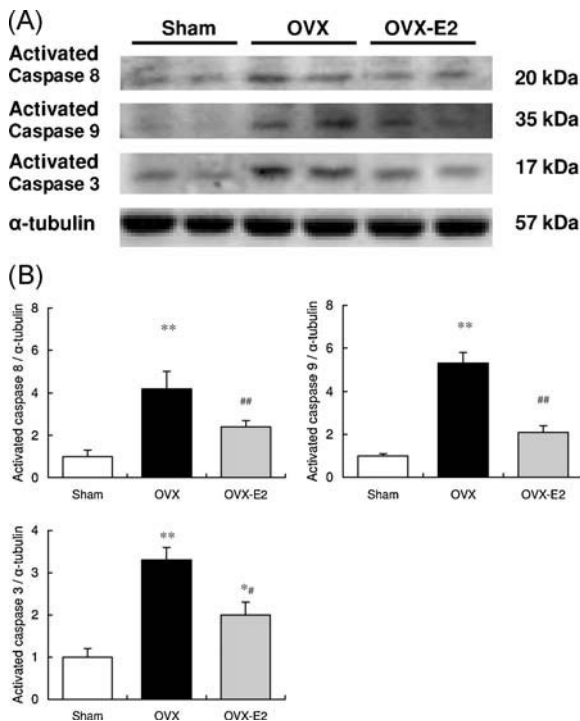
(A)

(B)

Figure 5. (A)The representative protein products of activated caspase 8, caspase 9, and caspase 3 extracted from the left ventricles of excised hearts in sham-operated rats (Sham), ovariectomized rats (OVX), and ovariectomized rats with 17β-estradiol (E2, 10 μg/kg/day, IP for 10 weeks) (OVX-E2) were measured by Western Blotting analysis. (B) Bars represent the relative protein quantification of caspase 8, caspase 9, and caspase 3 on the basis of α-tubulin and indicate mean values $\pm$ SD (n = 6 in each group). $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences from Sham group. $^{#}P < 0.05$, $^{##}P < 0.01$, significant differences from OVX group.

DISCUSSION

Our main findings can be summarized as follows: (1) chronic 17β-estradiol (E2) treatment prevents the increased heart weight, abnormal myocardial architecture, and more TUNEL-positive apoptotic cells after ovariectomy. (2) chronic 17β-estradiol (E2) treatment prevents bilateral ovariectomy-induced Fas receptor dependent apoptotic pathways, the evidence for which is based on the attenuation of TNF-α, Fas ligand (Fas L), Fas death receptors (Fas), Fas-associated death domain (FADD), activated caspase 8, and activated caspase 3 in OVX-E2 relative to OVX. (3) chronic 17β-estradiol (E2) treatment prevents bilateral ovariectomy-induced cardiac mitochondria-dependent apoptotic pathway, the evidence for which is based on the attenuation of t-Bid, Bax, Bax-to-Bcl2 ratio, Bax-to-Bcl-XL ratio, activated caspase 9, and activated caspase 3 in OVX-E2 relative to more increases in OVX. Our hypothesis proposed that cardiac Fas-dependent and mitochondria-dependent apoptotic pathways after ovariectomy can be prevented via chronic administration of 17β-estradiol (E2). (Figure 6)

A previous study suggested that the middle-aged ovariectomized rats have the potential to be good models

for the effect of ovariectomy and estrogen replacement on associated bone and cardiovascular changes [29]. Ovariectomized rats were reported to be involved in the menopause-related changes in biochemical and physiological functions, i.e. decreased levels of progesterone and estrogen [30], increased cardiovascular abnormalities [31-32], and enhanced rate of bone loss [33]. Since increased cardiac apoptosis was found in obese rats [11-12], ovariectomy-induced weight gain may potentially be a confounder in ovariectomy-induced cardiac apoptosis. The bilateral ovariectomies ''OVX'' in the current design not only impact female hormonal system but also impact female multiple systemic physiology such as weight gain and increased inflammation [34]. Endogenous estradiol is enzymatically converted to several nonestrogenic metabolites, and some of these metabolites induce potent biological effects [27]. Therefore, in the current experimental design, we have to add a cautious note that any detrimental effect of ovariectomy or any preventive effect of 17β-estradiol (E2) on hearts cannot be isolated to one specific factor but may be affected directly or indirectly by multiple factors, such as weight changes, ER receptors, ER-independent mechanism, estradiol metabolites, progesterone, oxidative stresses, inflammatory status, or unclear interacting factors.

The incident of heart failure in post-menopausal women was over 4-fold greater than that of women without menopause from 38283 participants during a 9-year follow-up [7]. A population-based cohort study showed that bilateral oophorectomies before 45 years old increase mortality compared with non-oophorectomic women [35]. Ovariectomized rats were previously reported to develop more extensive cardiac remodeling, characterized by significantly greater left ventricular hypertrophy and a substantial increase in left ventricular dilatation relative to non-ovariectomized rats [36]. In the current study and our previous study [15], cardiomyopathic changes, such as abnormal myocardial architecture, enlarged interstitial space, and cardiac widely dispersed apoptotic cells appear to be increased in ovariectomized rats.

Anti-apoptotic or protective effects of 17β-estradiol treatment on hearts were studied by using ischemic heart models in most of previous studies. Chronic 17β-estradiol treatment (7.5 mg/90 days) prevents post-myocardial infarction remodeling, such as dilatation, systolic dysfunction and diastolic dysfunction, in the rat left ventricules [37]. Pre-perfusion of 100 nM 17β-estradiol after 30 and 60 min stop-flow heart ischemia in Langendorff-perfused female rat hearts prevented the loss of cytochrome *c* from mitochondria, reduced activation of caspase-3-like activity and decreased DNA strand breaks in the nuclei of cardiomyocytes [38]. However, very limited information regarding the protective effects of 17β-estradiol on cardiac apoptosis or heart failure after post-menopause or bilateral oophorectomy in women was available. In current study, chronic 17β-estradiol treatment prevents bilateral ovariectomy-induced Fas receptor dependent apoptotic pathways, the evidence for which is based on the attenuation of TNF-α, Fas ligand, Fas death receptors, Fas-associated death domain, activated
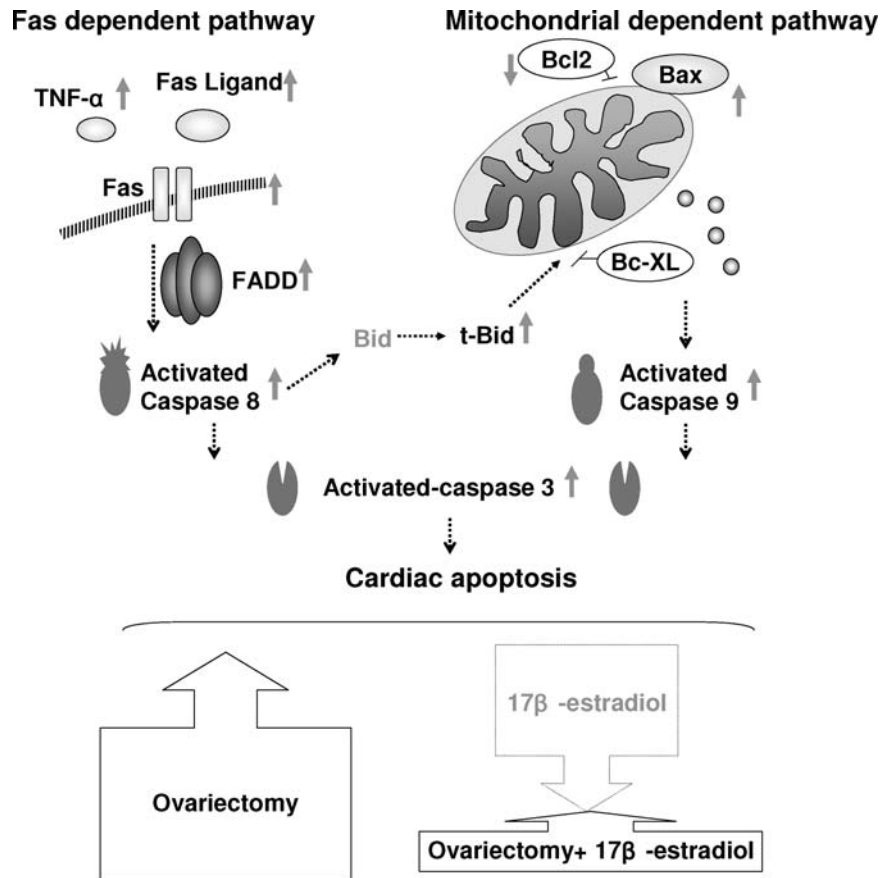
Figure 6. Our proposed major hypothesis: chronic 17β-estradiol (E2) treatment prevents ovariectomy-induced cardiac Fas-dependent and mitochondrial-dependent apoptotic pathways. After ovariectomy (bottom left arrow-ovariectomy), cardiac Fas-dependent apoptotic pathway appears to be increased due to increases in TNF-α, Fas, activated caspase-8, and activated caspase-3 as well as cardiac mitochondrial-dependent apoptotic pathway appears to be increased in OVX due to increases in t-Bid, Bax, Bax/BclXL, Bax/Bcl2, activated caspase-9, and activated caspase-3. Chronic 17β-estradiol treatment prevents ovariectomy-induced cardiac Fas-dependent and mitochondrial-dependent apoptotic pathways (bottom right arrow-ovariectomy plus 17β-estradiol (E2)).

caspase 8, and activated caspase 3. Chronic 17β-estradiol treatment prevents bilateral ovariectomy-induced cardiac mitochondria-dependent apoptotic pathway, the evidence for which is based on the attenuation of t-Bid, Bax, Bax-to-Bcl2 ratio, Bax-to-Bcl-XL ratio, activated caspase 9, and activated caspase 3. Therefore, our findings strongly suggest that chronic 17β-estradiol treatment did attenuate ovariectomy-induced cardiac Fas-dependent and mitochondrial-dependent apoptosis. Besides, the Bcl-2 family member t-Bid is one of the key components of main intracellular molecule signaling from Fas to mitochondrial apoptotic pathway [20,39]. Therefore, our finding also implys that 17β-estradiol also prevents molecule signaling, t-bid, from Fas to mitochondrial apoptotic pathway. Since cardiac tissues are difficult to be extracted from menopausal women hearts, the current ovariectomized animal model should provide an important mechanism for explaining the apoptosis-related cardiac diseases in women with the removal of ovaries or decline of female ovarian hormones. If 17β-estradiol did prevent cardiac apoptosis in post-menopausal women, the certain components of 17β-estradiol may potentially prevent the possible development of heart failure and sudden cardiac death. Menopausal women should be highly aware of the progressive development in cardiac abnormality and should actively promote heart health. Our current findings suggest that maintaining normal levels of 17β-estradiol is important to prevent ovariectomy-induced cardiac apoptotic pathways. The apoptotic pathways might provide one possible mechanism to interrupt the development of heart failure or cardiac apoptosis-related diseases in post-menopausal women. Of course, further therapeutic or clinical studies are required to clarify the beneficial effects of treatments or possible mechanisms in post-menopause-related heart abnormalities.

CONFLICT OF INTEREST

No conflict of interest.

ACKNOWLEDGEMENTS

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