行政院國家科學委員會補助專題研究計畫成果報告

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※ 雌性素防止心肌肥大及猝死機轉探討 ※

※Protective Mechanisms of 17β-Estradiol on the Hypertrophy※
※of Left Ventricle Induced by Coarctation of Abdominal Aorta※
※in Female Rats ※

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一、中文摘要

本研究發現雌性素，對心臟確實具有保護作用。實驗中將大白鼠分為兩組，一為卵巢完整（ sham ），另一為 3 週卵巢切除(OVX)，同時再將各組腹動脈結紮四天及腹動脈結紮八天或不結紮。並分別在大白鼠腹動脈結紮前四天，前一天，及結紮後三天，注射雌性素（ 17-β-E2，100ng/kg B.W.）。同時由心臟外觀，心臟全重及左心室重與體重的比例及 matrix-metalloproteases 含量，作為手術成功的指標。再進一步探討，insulin-like growth factor-1 (IGF-1)，insulin-like growth factor-1 receptor (IGF-1R)和 cytochrome-c-oxidase(COX vb)基因的表現差異及其相關訊息傳遞途徑，包含心肌生存途徑；PI3K-Akt，非心肌細胞增生相關途徑；MEK-ERK，及可能之病理性肥大途徑；calcineurin-NFAT3 的蛋白表現量與雌性素存在與否關係，並比較內生性雌性素及外生性雌性素補充對心臟影響的差異。結果顯示，內生性雌性素，在動脈阻塞下，對心肌功能維護相當重要。且雌性素保護心肌的機轉，與促進心肌生存途徑及非心肌細胞增生途徑，及壓制病理性肥大途徑有關。而雌性素的存在與否，並直接影響動脈結紮動物心肌中 IGF-I，IGF- I R 及 COX vb 的基因表現。唯獨較長期八天結紮動物，明顯造成心肌病變發生，額外補充雌性素失效且內生性雌性素的缺乏更加速病徵。

關鍵詞：雌性素、心肌細胞、腹動脈結紮

Abstract

To determine the effects of 17β-estradiol (E2) in overcoming the cardiac after-loading and prevention of cardiac fibrosis in rats. Moreover, the activities of the signaling molecules in cardiomyocytes survival, non-cardiomyocytes proliferation and pathological hypertrophy pathways were also examined to investigate the role of E2 in the cardiac protective mechanism. Sprague-Dawley rats were ovariectomized (OVX) one month before complete coarctation of the abdominal aorta (COX) with or without E2 treatment (100 ng/kg) and sacrificed 4 or 8 days later. Steady-state mRNA levels of IGF-I, IGF-IR, and cytochrome-c oxidase (Cox vb) were analyzed by dot blotting assay. Signaling proteins, matrix metalloproteinases (MMP-2, MMP-9) activity and cardiac fibrosis was analysed by Western blotting, zymography protease assay and histological examination, respectively. On day 4 coarcted animals, the heart weight and left ventricular weight, the latent form of MMP-2 in hearts of the rats with or without ovary intact significantly increased while these changes were reversed after E2 treatment. Moreover, the ovary intact animals overcame the hypertrophic effects and the consumption of MMP-2 on day 8. However, these effects were not restored in OVX animals and more fibrosis occurred. The activities of PI3K-Akt for cardiomyocyte survival and MEK-ERKs for non-cardiomyocytes proliferation pathways were significantly increased on day 4 whereas down-regulation was only observed on day 8 in OVX animals. Similarly, the steady-state mRNA expressions of IGF-I, IGF-IR, and Cox vb were elevated on day 4 in ovary intact animals and down-regulated on day 8 in OVX animals. The calcineurin/NFAT-3 pathway was suppressed on day 4 but elevated on day 8 in OVX animals. These findings indicate the up-regulation of IGF-I signaling and suppression of calcineurin/NFAT-3 pathway may

be plausible mechanisms for the cardiac protective effects of E2.

Key words: Abdominal Aorta Coarctation, 17β-Estradiol, Calcineurin/NFAT-3 pathway IGF-I signaling, Left Ventricle Hypertrophy

二、緣由與目的

Although the incidence of cardiovascular diseases in premenopausal women is about 50% of their male counterparts, this sexual difference becomes less apparent after the menopausal age [1]. These phenomena have been attributed to the significant reduction in the level of estrogens among the postmenopausal women [2]. Moreover, estrogen therapy to postmenopausal patients has been reported to be effective in reducing recurrent events and elevating survival rate [3]. For males with coronary artery disease, administration of estrogen of low dosages may prolong the duration of exercise and prevent abnormalities in electrocardiographs [4]. These clinical findings signify the cardiovascular protective effects of estrogen. However, the mechanisms of these direct effects of to cardiomyocytes remain unclear.

The principal estrogen secreted by the ovary and the most potent estrogen is 17β-estradiol (E2). The mechanism of estrogen action in target tissues involves binding to a nuclear steroid receptor and enhancement of the transcription of mRNA, which in turn causes increased protein synthesis in the cytoplasm [6-13]. In the cardiomyocytes, two receptors of estrogen have been identified: estrogen receptor α (ERα) [14, 15] and estrogen receptor β (ERβ) [16]. ERα may interact with insulin-like growth factor-1 receptor (IGF-IR) and enhance the phosphorylation of ERK1/2. This interaction not only accelerates the interaction between estrogen and ERα but also the IGF-IR-related signal transduction pathways. The cross-talking and/or positive feedback between E2 and IGF-I pathways result in the potentiation MEK-ERK signal pathway [17-19].

In addition to ERα, there is a 45-kDa ERα-like receptor on the membrane of the human vascular endothelial cells. This receptor may mediate genetic (slow) or non-genetic (rapid) intracellular signaling [20]. It has been reported that IGF-I inhibits apoptosis through the phosphatidylinositol 3'-kinase (PI3K) and mitogen-activated protein kinase pathways [21]. The PI3K pathway has been demonstrated in determining the heart size in mice [22]. Therefore, the PI3K-Akt/PKB pathway via IGF-IR may involve in the physiological hypertrophy of the cardiomyocytes. Moreover, IGF-I may activate Ras-Raf-MEK-ERK to enhance hypertrophy of non-cardiomyocytes [23]. Therefore, the interaction between E2-ER and IGF-1-IGF-IR may play an important role in the protection of cardiomyocytes.

Pathological hypertrophy of the cardiomyocytes is the main cause of sudden cardiac death. However, its mechanism at the molecular level remains unclear. It has been reported that activation of Ca^{2+} may induce the calcineurin-NFAT pathway and in turn enhance hypertrophy or apoptosis of cardiomyocytes [24]. Calcineurin (protein phosphatase 2B or PP2B) is a heterodimer [25]. At high concentration of Ca^{2+}, changes in the structure calcineurin B may lead to the activation of calcineurin [26]. After phosphorylation, calcineurin A may participate in the regulation of the transcription of the T cell growth factor (interleukin-2) [27, 28] and translocate nuclear factor activation transcription-3 (NFAT-3) into the nucleus for gene expression [29]. Calcineurin may also regulate mitogen-activated protein kinase (MAPK) pathway [30] and affect the activity of PKCα, PKCθ, and JNK [31]. These changes may induce pathological hypertrophy of cardiomyocytes [32], loss of cytochrome c [33], and dephosphorylation of bad [34]. Since E2 analogue, genistein may result in reduction of Ca^{2+} influx in cardiomyocytes [36], it may play a role in the prevention of these changes.

In cardiomyocytes, IGF-I is a survival factor that activates the PI3K-Akt/PKB pathway via IGF-IR [21] and the

Ras-Raf-MEK-ERK through IRS-1 [35] in physiological hypertrophy and hyperplasia, respectively. Pathological hypertrophy may be caused by the Ca^{2+} influx and calcineurin/NFAT-3 pathway activation [32]. In this study, we determined the effects of E2 in overcoming the cardiac after-loading promoted by experimental hypertension and prevention of cardiac fibrosis in rats ovariectomy and complete abdominal aorta coarctation with or without E2 treatment. Moreover, the activities of the signaling molecules in these three pathways were also examined to investigate the role of E2 in the cardiac protective mechanism.

三、研究方法

2.1. Materials

Antibodies against calcineurin, Akt, MEK 1, Bad, Bcl-2, PI3K, PKCα were purchased from Transduction Laboratories (Lexington, KY), against cytochrome c from R&D Systems (Minneapolis, MN), against IGF-IR alpha-subunit from Upstate Biotechnology (Lake Placid, NY), against ERK 1 and α-tubulin from Neo Markers (Fremont, CA), against ERα from Affinity Bio Reagents (Golden, CO), against NFAT-3 and IGF-I from Snata Cruz Biotechnology (Santa Cruz, CA), against Akt [pS473], ERK1/2 [pTpY185/187], and JNK [pTpY183/185] from Bio Source (Camarillo, CA). Rabbit anti-goat IgG, goat anti-mouse IgG, goat anti-rabbit IgG, were from Antibodies Incorporated (Davis, CA).

2.2. Animal Preparation

Female Sprague-Dawley rats (200 g) were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. These animals were maintained in a temperature-controlled environment (24 ± 1°C) and illuminated for 12 h daily (5 AM - 5 PM) and fed with commercial pellets and water ad libitum. Under light ether anesthesia, half of these animals were bilaterally ovariectomize (OVX) and the remaining ones were sham-operated. One month later, cardiac hypertrophy was induced in half of the rats in each group by complete coarctation of the abdominal aorta between the origins of the renal arteries and the remaining one were sham-operated. The rats were scarified by decapitation on day 4 or day 8 after the operation. Each rat was treated with 17β-estradiol (100 ng/kg) or sesame oil on day 4 and day 1 before the operation. An additional dosage of 17β-estradiol or sesame oil was administered to each animal on day 4 among those sacrificed on day 8 post-operation. The group received sham operations and treated with sesame oil was considered to be the control. All experiments were carried out after approval of the University ethics committee for the use of experimental animals and conform with the *Guide for Care and Use of Laboratory Animals.*

2.3. Macroscopic and Histological Examination

The rats were weighed before sacrifice. After removal from the thoracic cavity, the heart was cleaned with dd H_2O and dried before taking the total heart weight. The left and right atrium and the right ventricle were then removed and the weight of left ventricle was determined. The ratios of the total heart weight and weight of left ventricle to body weight were calculated. Histological sections were prepared using hematoxylin and eosin stain.

2.4. Tissue Extraction

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples in a PBS buffer (0.14 M NaCl, 3 mM KCl, 1.4 mM KH_2PO_4, 14 mM K_2HPO_4) at a concentration of 1 mg tissue/10 μl PBS for 5 min. The homogenates were placed on ice for 10 min and then centrifuged at 12,000 rpm for 30 min. The supernatant was collected and stored at -70°C for further experiments.

2.5. Isolation of Heart Mitochondria

After adding 1 ml PBS to 0.1g left ventricular tissues, the tissues were minced with scissors and homogenized at 4°C with a Model PT 10/35 Polytron homogenizer (setting 11, Brinkmann Instruments, Westburg, NY) for 5 min before centrifuging at 2,300 rpm for 10 min in a Beckman J20.1 centrifuge, the pellet discarded, and the supernatant was then centrifuged at 9,400

rpm for 15 min. The mitochondrial pellet was resuspended to 200 μl PBS. All steps were done at 0-4℃.

2.6. Protein Contents

Protein contents of the cardiac tissue extracts were determined by the Bradford protein assay [48] using the protein-dye kit (Bio-Rad, Richmond, CA, USA). Coomassie brilliant blue G-250 was used for staining and a commercial available bovine serum albumin (Sigma Chemical, St. Louis, MO) was employed as a standard. Changes in optical density were monitored at 595 nm.

2.7. Gelatin Zymography Protease Assay

The cardiac tissue extracts (40 μg) were mixed thoroughly with a suitable volume of PBS buffer and 4 μl of dye. Gelatin zymography analysis was carried out by loading 20μl of the extracts 0.1% gelatin- and 8% SDS-PAGE and ran by electrophoresis at 140 V for 2.5 h. The gels were washed in a 2.5% Triton X-100 solution with shaking for 30 min and then incubated in 50 ml reaction buffer (40 mM Tris-HCl, pH 8.0; 10 mM CaCl2, 0.01% NaN_3) at 37°C for 12 h before staining with 0.25% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid for 1 h. Quantitative analysis was preformed after discoloring the stain in a destaining solution (10% acetic acid, 20% methanol) twice for 30 min.

2.8. Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 9.5% polyacrylamide gels [49]. After mixing a sample of cardiac tissue extracts (40 μg) with a suitable volume of PBS buffer and 4 μl bromphenol-blue dye, the mixture was heated at 95°C for 10 min and then rapidly placed in an ice bath. The mixture was spin-down in a centrifuge and loaded onto slab polyacrylamide gels before electrophoresing at 110 V for 90 min. The gels were then equilibrated in a transfer buffer (pH 8.3) containing Tis base 3 g, glycine 14.4 g and methanol 200 ml. Proteins were transferred onto nitrocellulose paper (Amersham, Hybond-C Extra Supported, 0.45 Micro) using a Hoefer Scientific Instruments Transphor Units at 150 mA for 2 h. The nitrocellulose papers were incubated at room temperature for 2 h in a blocking buffer containing 100 mA for 14 hour. Nitrocellulose papers were incubated in a blocking buffer containing 100 mM Tris-HCL (pH 7.5), 0.9% (w/v) NaCL, 0.1% (v/v) fetal bovine serum at room temperature for 2 h.

Antibodies against IGF-I (1:250), IGF-IR (1:125), PI3K (1:1000), PKBα/Akt (1:400), bad (1:1000), bcl-2 (1:500), MEK 1 (1:1000), ERK (1:1000), PKCα (1:400), NFAT-3 (1:200), cytochrome c (1:400), cytochrome c oxidase (1:400), α-tubulin (1:500),ERα (1:400), Anti-PKB/Akt [pS^{473}] (1:400), Anti-ERK 1&2/MAPK [$pTpY^{185/187}$] (1:400), Anti-JNK 1&2/SAPK [$pTpY^{183/185}$] (1:400) were diluted to the corresponding concentrations in an antibody binding buffer containing 100 mM Tris-HCl (pH 7.5), 0.9% (w/v) NaCL, 0.1% (v/v) Tween-20, and 1% (v/v) fetal bovine serum. These mixtures were incubated performed at room temperature for 3.5 h. Immunoblots were washed in triplicate in 50 ml blotting buffer for 10 min and then immersed in the secondary antibody solution containing alkaline phosphatase goat anti-rabbit IgG (Promega) for 1 h before dilution 1000-fold in the binding buffer. The filters were washed in triplicate in the blotting buffer for 10 min. Color development was presented in a 20-ml mixture consisting of 7 mg nitro blue tetrazolium, 5 mg 5-bromo-4-chloro-3-indolyl-phosphate, 100mM NaCl, and 5 mM $MgCl_2$ in 100 mM Tris-HCl (pH 9.5).

2.9. RNA Extraction

Total RNA was extracted using the Ultraspec RNA Isolation System (Biotecx Laboratories, Inc.) according to instructions of the manufacturer. Each heart was thoroughly homogenized in 1 ml Ultraspec reagent/100 mg tissue with the Polytron homogenizer. The homogenates were washed twice with 70% ethanol by gentle vortexing. RNA precipitates were then collected by centrifugation at 12,000 *g* and dried under vacuum for 5-10 min before dissolving in 50μl diethylpyrocarbonate-treated water, and then incubating at 55-60°C for 10-15 min.

2.10. RNA Dot Blotting

RNA dot blotting was used for the hybridization and detection of IGF-I, IGR-IR, and Cox vb mRNAs according to the method described in our previous study [50]. The corresponding digoxigenin (DIG)-labeled antisense RNA probes were prepared from pGEM-1 containing a *Bam*H1-*Eco*R1 956-bp insert consisting of exon 3 and flanking intron sequences from the rat IGF-I gene [51], pGEM-7Zf(+) containing a 500-bp insert consisting of mouse kidney cDNA encoding Cox subunit Vb (supplied by Dr. Dennis E. Buetow, University of Illionois, Urbana-Champaign, IL, USA), the pTRI-IGFR-human transcription template containing a 236-bp cDNA fragement of the human IGF-IR gene spaning exons 8-7 (Ambion, Austin, TX, USA).

2.11. Statistical analysis

Data were expressed as mean ± SEM. Differences among the groups were determined by the unpaired Student's *t* test. $P < 0.05$ was considered to be statistically significant.

四 結果

3.1. Histological Changes in the Heart

On day 4, fibrosis in the heart was observed only in two rats (50%) with OVX and COX. However, no apparent macroscopic changes in the hearts were observed in the remaining groups. On day 8, fibrosis was not only observed in OVX + COX animals with (71%) and without (67%) E2 treatment but also in ovary-intact COX animals with (40%) and without (43%) E2 treatment (Table 1).

3.2. Changes in Heart Size

On day 4, ratio of total heart weight or left ventricle weight to body weight was found to be significantly higher in the animals with COX ($P < 0.01$) or OVX + COX ($P < 0.05$) than the control. However, only ratio of left ventricle weight to body weight and that of total heart weight to body weight were found to be significantly higher in the corresponding groups with E2 treatment. Although significantly higher ratio of total heart weight to body weight was found in the COX rats ($P < 0.05$) on day 8, the two ratios were found to be significantly higher in the rats with OVX + COX with ($P < 0.05$) or without ($P < 0.01$) E2 treatment (Fig. 1).

3.3. Changes in MMP-2

Levels of MMP-2 were found to be significantly higher in the ovary intact or OVX rats with COX but without E2 treatment ($P < 0.05$) on day 4. However, significantly lower levels were observed in the sham-operated animals with COX without E2 treatment or those with OVX + COX with or without 17β-estradiol treatment ($P < 0.05$) on day 8 (Table 2).

3.4. Changes in the Signaling Proteins

On day 4 after COX, levels of the signaling proteins in the cardiomyocyte survival pathway (IGF-I, IGF-IR, PI3K, Akt, Akt-P, bcl2, cytochrome-c, and cytochrome-c oxidase) in animals with and without OVX were found to be higher than the control whereas the level of bad decreased. Similar increasing trends were also found in the non-cardiomyocyte proliferation pathway (MEK, ERK 1/2 , ERK-P). However, in the calcineurin/NFAT-3 pathway, there was no significant changes in calcineurin, NFAT-3, PKCα, and JNK-P among the animals with ovary intact whereas calcineurin had an increasing trend in those with OVX (Fig. 2; Table 3).

Increasing trends in the levels of the signaling proteins in the cardiomyocyte survival pathway and non-cardiomyocyte proliferation pathway were observed in rats with ovary intact on day 8. The level of bad was also found to be decreasing. However, calcineurin had an increasing trend. Among the OVX rats, the survival and proliferation pathways were observed to have decreasing trends. Moreover, the level of calcineurin, NFAT-3, PKCα, and JNK-P increased significantly (Fig. 3; Table 4).

3.5. Changes at the Transcriptional Level

On day 4 after COX, significantly higher levels of steady-state mRNA expression of IGF-I ($P < 0.05$), IGF-IR ($P < 0.05$), and cytochrome-c oxidase (Cox vb, $P < 0.01$) were found in the sham-operated animals. These levels became higher in the sham group with E2 treatment. However, no

significant changes in the expression levels of IGF-I, IGF-IR, and Cox vb in the OVX rats with or without E2 treatment (Fig. 4). In the rats with ovary intact, no significant changes in the expression level were found in these levels of expression on day 8. Moreover, significantly lower levels ($P < 0.01$) were found in the OVX ones even with E2 treatment ($P < 0.05$) (Fig. 5).

五 討論

Estrogen has been reported to be effective in suppression or delay left ventricular hypertrophy in clinical studies [2, 5]. However, inconsistent findings on the effects of estrogen have been obtained in different animals or induction models. E2 may suppress or reduce experimental left ventricular hypertrophy in OVX SHHF/$M_{cc\text{-}facp}$ rats [37] or sinoaortic denerrated rats [38]. However, left ventricular hypertrophy has been reported in female rabbits treated with E2 (0.15 μg/kg/day) [39]. After treating OVX goats with E2 at a dose of 0.6 mg/kg/week, left ventricular hypertrophy may also be induced.

In this study, we employed the ratios of total heart weight to body weight and weight of left ventricle to body weight as indicators for the heart and left ventricular sizes, respectively. Increase in these ratios has been demonstrated in COX rats. Moreover, fibrosis of cardiomyocytes was also observed in these rats [40, 41]. These changes may be attributed to damages caused by the experimental hypertension and may lead to the hypertrophy and death of cardiomyocytes. After experimental hypertension by COX, we found that the heart/ventricular size was significantly higher in COX rats of 4 days but no cardiac fibrosis was observed in the groups with endogenous E2 (ovary intact) and decrease in the heart/ventricular size was observed in rats with E2 treatment. In addition, E2 treatment may prevent the rats from cardiac fibrosis, since 50% of the OVX rats without the treatment were found to have cardiac fibrosis. Moreover, E2 treatment was also found to be effective in recovering the heart/ventricular size of OVX rats. These finding indicate that E2 may delay the occurrence of cardiac hypertrophy and prevent fibrosis in the heart.

Contrary to the findings on day 4, only the ratio of total heart weight to body weight in animals with ovary intact was found to increase significantly on day 8, but E2 treatment remained to have improvement to these ratios. Owing to the deficiency in the endogenous E2, cardiac fibrosis occurred more severely in OVX rats with or without E2 treatment. Their heart size was also significantly increased. Moreover, no improvement was observed in the OVX ones with E2 treatment. These findings suggest that only exogenous E2 is not enough to reverse the damages in heart induced by long-term pressure over-loaded in the OVX animals. However, the cardiac disorders in the ovary intact ones might be reversed by E2 treatment.

There is an association between the turnover of MMP-2 or collagens and remodeling of the rat right and left ventricles in experimental hypertension [41]. The remodeling progresses immediately after myocardial damage with increase in the level of collagenases and cardiac hypertrophy [42]. Since MMP-2 is a member of gelatinase A family, this enzyme has the capability in the hydrolysis of collagens Ⅰ, Ⅳ, Ⅴ and Ⅶ [43]. Therefore, the level of MMP-2 should elevate during cardiac remodeling. It has been reported that the severity of cardiac fibrosis may become significantly high on day 8 after COX [44]. Necrosis of the cardiomyocytes and replacement with fibroblasts may lead to on an accumulation of collagens. In the COX animals sacrificed on day 4, we found cardiac hypertrophy with an increase in the level MMP-2. However, the level remained relatively constant among those with E2 treatment. The decrease in the level MMP-2 in rats with intact ovary sacrificed on day 8 lead to the increase in severity of fibrosis. This change was eliminated by the treatment with E2. However, severe fibrosis was observed in OVX rats with significant decrease in the level of MMP-2. This change became

irreversible even after E2 treatment. These findings indicate that E2 may affect the remodeling of the heart and the metabolism of the collagen. Moreover, endogenous E2 may have an association with MMP-2 and the cardiac protective mechanism.

In order to investigate the effects of endogenous and exogenous E2 in the regulation of physiological hypertrophy, proliferation, and pathological hypertrophy, we employed Western blotting to determine the levels of signaling proteins in the three pathways. On day 4 after COX, the levels of signaling proteins in the cardiomyocyte survival pathway were found to have significant increasing trends in the animals with or without ovary intact. The increasing level became even higher in those with E2 treatment. Moreover, the levels of cytochrome-c and cytochrome-c oxidase in mitochondria were also significantly increased in these animals. Similar changes were observed in the non-cardiomyocyte proliferation pathway in the animal with or without ovary intact. These findings indicate that high-level heart remodeling and physiological cardiac hypertrophy occurred in the animals at this stage. Moreover, the levels of the signaling proteins in the calcineurin/NFAT-3 remained relatively constant in the animals with ovary intact. This phenomenon may be due to the function of endogenous E2. However, the level of calcineurin was found to be increased in the OVX rats whereas no significant changes were observed in NFAT-3, PKCα, and phosphorylated JNK. Although these findings suggest an increasing trend for the occurrence of pathological hypertrophy, the levels of NFAT-3, PKCα, and phosphorylated JNK may be regulated by other pathways such as the phosphorylation of NFAT-3 through MEK-ERK or the influence to the L-type Ca^{2+} channel by E2.

Although similar findings in these pathways were observed in animals with ovary intact on day 8 after COX, survival and proliferation pathways were downregulated in the OVX animals and the levels of calcineurin, NFAT-3, PKCα, and JNK were found to have a significantly higher level. The activity downregulated of the survival pathway in OVX animals was not changed by E2 treatment. In addition, the levels of MEK and ERK were reduced as well and the levels of calcineurin, NFAT-3, and phosphorylated JNK were significantly increased. These findings indicate that E2 may protect the cardiomyocytes by the up-regulation of the survival and proliferation pathways and suppression of the calcineurin/NFAT-3 pathway to reduce the pathologic changes in these cells.

Although we have obtained some information on the cardiac protective effects of E2 at the molecular level, it is important to determine the changes at the transcriptions level. It has been reported that the interaction of IGF-I and IGF-IR may activate IGF-IR, which may in turn enhance the phosphorylation of PI3K [45]. The activated PI3K enhances the phosphorylation of Akt [46]. Akt-P then regulates the levels of bcl-2 and bad to control the viability and apoptosis of cardiomyocytes [47]. Therefore, we determined the expression levels of mRNA in IGF-I, IGF-IR, and the oxidative phosphorylation key enzyme, Cox vb. Although E2 treatment was found to be effective in enhancing these expression levels in animals with ovary intact on day 4 after COX, this hormone may have litter effects in changing the decreasing trends in these levels in OVX rats on day 8. Based on these findings at the macroscopic, molecular, and transcriptional levels, the cardiac protective effects of E2 may be mediated through the up-regulation of the IGF-I signaling and suppression of the calcineurin/NFAT-3 pathway.

六、計畫成果自評

實驗進行順利，成果顯著。

七、參考文獻

[1] Stubblefield PG. Postmenopausal estrogen and cardiovascular disease. New Engl J Med 1987; 316:342-343

[2] Katz AM. Cardiomyopathy of overload: A major determinant of prognosis in congestive heart failure. New Engl J Med

1990; 322:100-110
[3] Grodstein F, Stampfer MJ. Estrogen for women at varying risk of coronary disease. Maturitas 1998; 30(1):19-26
[4] Grossman W. Diastolic dysfunction in congestive heart failure. New Engl J Med 1991; 325:1557-1564
[5] du Cailar G, Ribstein J, Pasquie JL, Mimran A. Determinant of left ventricular hypertrophy in the hypertensive woman. Influence of hormone replacement therapy for menopause. Arch Mal Coeur Vaiss 1999; 92(8):975-977
[6] Kumar V, Green S, Stack G, Berry M, Jin J-R, Chambon P. Functional domains of the human estrogen receptor. Cell 1987; 51:941-951
[7] Evans RM. The steroid and thyroid hormone receptor super-family. Science 1988; 240:889-895
[8] Kurebayashi J, Otsuki T, Kunisue H, Tanaka K, Yamamoto S, Sonoo H. Expression levels of estrogen receptor-α, estrogen receptor-β, coactivators, and corepressors in breast cancer. Clin Cancer Res 2000; 6:512-518
[9] Tora L, White CB, Tasset O, Webster N, Scheer E, Chambon P. The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell 1989; 59:477-487
[10] Hyder SM, Stancel GM. In vitro interaction of uterine estrogen receptor with the estrogen response element present in the 3'-flanking region of the murine c-*fos* protooncogene. J Steroid Biochem Mol Biol 1994; 48:69-79
[11] Bhargava V, Kell DL, Rijn M, Warnke RA. Bcl-2 immunoreactivity in breast carcinoma correlates with hormone receptor positivity. Am J Pathol 1995; 145:535-540
[12] Kraus WL, Mcinerney EM, Katzenellenbogen BS. Ligand-dependent, transcriptionally productive association of the amino- and carboxy-terminal regions of a steroid hormone nuclear receptor. Proc Natl Acad Sci USA 1995; 92:12314-12318
[13] Salmi A, Ammala M, Rutanen EM. Proto-oncogenes c-*jun* and c-*fos* are down-regulated in human endometrium during pregnancy: Relationship to oestrogen receptor status. Mol Hum Reprod 1996; 2:979-984
[14] Green S, Walter P, Kumar V et al. Human oestrogen receptor cDNA sequence expression and homology to v-erb-A. Nature 1986; 320:134-139
[15] Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. Sequence and expression of estrogen receptor complementary DNA. Science 1986; 231:1150-1154
[16] Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 1996; 93:5925-5930
[17] Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 1995; 270:1491-1494
[18] Richards RG, Diaugustine RP, Petrusz P, Clark GC, Sebastian J. Estradiol stimulates tyrosine phosphorylation of the insulin-like growth factor-1 receptor and insulin receptor substrate-1 in the uterus. Proc Natl Acad Sci U S A. 1996; 93:12002-12007
[19] Kahlert S, Nuedling S, van Eickels M, Vetter H, Rainer M, Grohe C. Estrogen receptor α rapidly activates the IGF-1 receptor pathway. J Biol Chem 2000; 275:18447-18453
[20] Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR. Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. Proc Natl Acad Sci USA 2000; 97:5930-5935
[21] Marcelina P, Alan RS, Derek L. Insulin-like growth factor 1 inhibits apoptosis using the phosphatidylinositol 3'-kinase and mitogen- activated protein kinase pathways. J Biol Chem 1997; 272:154-161.
[22] Tetsuo S, Peter MK, Pamela SD et al. The conserved phosphoinositide

3-kinase pathway determines heart size in mice. EMBO J 2000; 19:2537-2548

[23] Claire AM, Felicity EBM, Bruce RW. Insulin receptor substrate-1 expression is regulated by estrogen in the MCF-7 human breast cancer cell line. J Biol Chem 2000; 275:12565-12571

[24] Tian-Li Y, Eliot HO, Robert RR Jr. Apoptosis: A potential target for discovering novel therapies for cardiovascular diseases. Chem Bio 1999; 1 3:474-480

[25] Anglister J, Grzesiek S, Wang AC, Ren H, Klee CB, Bax A. 1H,13C,15N nuclear magnetic resonance backbone assignments and secondary structure of human calcineurin B. Biochemistry 1994; 33:3540-3547

[26] Kakalis LT, Kennedy M, Sikkink R, Rusnak F, Armitage IM. Characterization of the calcium-binding sites of calcineurin B. FEBS Lett 1995; 362:55-58

[27] Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK 506. Immunol Today 1992; 13:136-142

[28] Crabtree GR, Clipstone NA. Signal transmission between the plasma membrane and nucleus of T lymphocytes. Annu Rev Biochem 1994; 63:1045-1083

[29] Liu J, Farmer JO, Lane WS, Friedman J, Weissman I, Schreiber SL Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-506 complexes. Cell 1991; 66:807-815.

[30] Sugden, PH. Signaling in myocardial hypertrophy: Life after calcineurin? Circ Res 1999; 84:633-646

[31] De Windt LJ, Lim HW, Syed H, Thomas F, Molkentin JD. Calcineurin promotes protein kinase C and c-*jun* NH2-terminal kinase activation in the heart. J Biol Chem 2000; 275:13571-13579

[32] Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998; 93:215-228

[33] Yang J, Liu X, Bhalla K et al. Prevention of apoptosis by bcl 2; release of cytochrome c from mitochondria blocked. Science 1997; 275:1129-1132

[34] Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F. Ca^{2+}-induced apoptosis through calcineurin dephosphorylation of BAD. Science Apr 1999; 284:339-343

[35] Hoshijima M, Sah VP, Wang Y, Chien KR, Brown JH. The low molecular weight GTPase Rho regulates myofibril formation and organization in neonatal rat ventricular myocytes. Involvement of Rho kinase. J Biol Chem 1998; 273:7725-7730

[36] Chiang CE, Chen SA, Chang MS, Lin CI, Luk HN. Genistein directly inhibits L-type calcium currents but potentiates camp-dependent chloride currents in cardiomyocytes. Biochem Biophys Res Commun 1996; 223:598-603

[37] Pelzer T, Shamim A, Wolfges S, Schumann M, Neyses L. Modulation of cardiac hypertrophy by estrogens. Adv Exp Med Biol 1997; 432:83-89

[38] Neyses L, Pelzer T. The biological cascade leading to cardiac hypertrophy. Eur Heart J 1995; 16, Suppl N:8-11

[39] Lim WK, Wren B, Jepson N, Roy S, Caplan G. Effect of hormone replacement therapy on left ventricular hypertrophy. Am J Cardiol 1999; 83(7):1132-1134,A9

[40] Borg TK, Terracio L. Interaction of the extracellular matrix with cardiomyocytes during development and disease. In:Stolte H, Kinne RKH, Bach PH eds. Cardiac Myocyte-Connective Tissue Interactions in Health and Disease, New York:S. Karger AG, 1990; 113-129

[41] Brilla CG, Pick R, Tan LB, Janicki JS, Weber KT. Remodeling of the rat right and left ventricles in experimental hypertension. Circ Res 1990; 67:1355-1364

[42] Janicki JS, Brower GL, Henegar JR, Wang L. Ventricular remodeling in heart failure: the role of myocardial

collagen. Adv Exp Med Biol 1995; 382:239-245
[43] Dollery CM, McEwan JR, Henney AM. Matrix metalloproteinases and cardiovascular diseas. Circ Res 1995; 77:863-880
[44] Weber KT, Brilla CG, Campbell SE. Regulatory mechanisms of myocardial hypertrophy and fibrosis: results of in vivo studies. Cardiology 1993; 81:266-273
[45] Datta SR, Brunet A, Greenberg ME. Cellular survival: A play in three Akts. Genes Dev 1999; 13:2905-27
[46] Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 2000; 407:538-541
[47] Campbell RA, Poornima B-N, Patel NM, Constantinidou D, Ali S, Nakshatri H. PI3K kinase/Akt-mediated activation of estrogen receptor alpha: A new model for anti-estrogen resistance. J Biol Chem 2001; 276:9817-9824
[48] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye· binding. Anal Biochem 1976 ;72:248-254.
[49] Laemmli UK. Cleavage of structure protein during the assembly of the head of bacteriophage T4. Nature 1970; 27:680-685.
[50] Huang CY, Kasai M, Buetow DE. Extremely-rapid RNA detection in dot blots with digoxigenin-labeled RNA probes. Genet Anal: Biomolec Engineer 1998; 14:104-112.
[51] Park GH, Buetow DE. Genes for insulin-like growth factor I and II are expressed in senescent rat tissues. Gerontology 1991; 37:310-316