



# 行政院國家科學委員會專題研究計畫成果報告

## 動情激素對大鼠心肌梗塞後之左心室再塑型和心律不整之影響 Effects of Estrogen on Left Ventricular Remodeling and Arrhythmias in Post-Myocardial Infarction Rats

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### 一、中文摘要

心肌梗塞後常誘發心肌細胞肥厚進而造成心律不整。內皮素-1對心肌肥厚之發展伴演重要角色。我們探討動情激素是否在心肌梗塞後，可以減少心肌肥厚，而此作用是否與減低內皮素-1有關。

在綁完左前降枝後，在去卵巢後之雌大鼠分配到補充或沒補充動情激素組四週後。發現動情激素可減少心肌細胞大小。若未補充動情激素，在心梗塞後內皮素增加4.5倍，若給予動情激素後，內皮素濃度明顯地下降。如此內皮素-1之變化也可經由免疫組織染色得證。而心律不整之分數未補充動情激素明顯地比補充者高。

我們結論：動情激素可經由降低局部內皮素-1而減少心肌肥厚程度。

**關鍵詞：**內皮素、動情激素、心肌梗塞、心室再塑型內皮素

### Abstract

Reactive cardiomyocyte hypertrophy after myocardial infarction is an important risk factor for arrhythmias. Endothelin (ET)-1 has been implicated in the development of cardiac hypertrophy. We investigated the effect of estrogen on ventricular hypertrophy during remodeling after myocardial infarction and whether the attenuated hypertrophic effect was via reduced regional ET-1 expression.

After ligation of the anterior descending

randomized to estrogen supplementation alone, induced myocardial infarction without or with supplemented estrogen for 4 weeks. Estrogen decreased cardiomyocyte sizes isolated by enzymatic dissociation at the border zone compared with without supplemented estrogen ( $2417 \pm 426$  vs.  $3264 \pm 516 \mu\text{m}^2$ ,  $P < 0.0001$ ). The myocardial ET-1 levels from the border zone were 4.5-fold higher ( $P < 0.0001$ ) in the vehicle group compared with sham group although plasma ET-1 was not significantly changed. The increased regional ET-1 levels can be inhibited after estrogen administration. Immunohistochemistry confirmed the localization of ET-1 mainly in the cardiomyocytes. Arrhythmic scores during programmed stimulation were significantly higher in the vehicle than those treated with estrogen.

**Conclusion.** The results of the present study suggest that estrogen administration after infarction can reduce the inducibility of ventricular arrhythmias as a result of attenuated cardiomyocyte hypertrophy through decreased tissue ET-1 level.

**Keywords:** Endothelin-1; Estrogen; Myocardial infarction; Ventricular remodeling.

### 二、緣由與目的

Cardiac remodeling was associated with myocardial hypertrophy and left ventricular (LV) dilation following myocardial infarction (MI) (1). On the cellular level, the hypertrophic phenotype is characterized by an increase in cell size. These changes in LV geometry contribute to the development of depressed cardiac performance, arrhythmias and sudden cardiac death (1). There is considerable evidence that electrophysiological changes associated with the hypertrophied myocardium (2). Hypertrophied myocardium has been shown to generate arrhythmias more readily than normal tissue. Agents with the regression of ventricular hypertrophy have been shown to decrease the susceptibility of ventricular arrhythmias (3).

Endothelin (ET) system plays a role in ventricular remodeling after myocardial infarction (MI) (4). ET-1, a potent growth-promoting peptide derived from endothelial cells, is also produced by cardiac myocytes (5). ET-1 acts as a key autocrine/paracrine mediator to trigger the hypertrophic signaling pathways by activation of extracellular signal-regulated kinase in myocardium (6). Plasma and tissue levels of ET-1 increases in acute MI are associated with a poor prognosis (7). The ET system is a promising candidate for cardiac remodeling after MI because ET-1 receptor blockade attenuates ventricular remodeling (4).

Epidemiological studies have shown controversial results whether estrogen administration may be beneficial in secondary prevention of cardiovascular morbidity and mortality. Some studies showed positive results (8-10). The other showed neutral (11) or negative results (12,13). The evidence is largely from observational studies rather than random-allocation trials, due to difficulties in

instituting the latter. The first prospective and randomized study on the subject (HERS, 13) showed no advantage of hormone replacement therapy in secondary prevention of cardiovascular mortality. However, the study has been criticized due to the addition of progesterone to the therapy regimen. Progesterone could display a number of potential adverse effects on the cardiovascular system that might overcome the beneficial influence of estrogens. The conflicting role of estrogen after myocardial infarction occurs not only in clinical trials but also in animal studies. Hugel et al (14) have demonstrated that estrogen had no significant effect on post-infarction remodeling in the rat model. However, Smith et al (15) showed in a similar study that estrogen provides a favorable ventricular remodeling and prolongs long-term survival. Various factors may have influenced the experimental studies such as infarct size and timing to start of treatment after MI. Because treatment prior to acute myocardial infarction is a virtual impossibility in most clinical situations, there has been a great deal of interest in agents favorable in ventricular remodeling. Estrogens have been proved to inhibit the effect of ET-1 on cellular function including cellular hypertrophy (15). Therefore, the study was aimed to elucidate the possible contribution of estrogens to cardiac phenotypic modulation by attenuation of ET-1 in rat hearts with similar infarct sizes and we explored the downstream functional significance of reduced ventricular hypertrophy by ventricular pacing in a rat myocardial infarction model.

### ≡ · Methodology

#### *Animals.*

Procedures for animal care, surgery, and euthanasia were approved by our institutional review committee for animal experiments.

Female Wistar rats that weighed about 200 g fed a normal sodium diet and offered tap water ad libitum before the study. They were kept in cages, 5 per cage, in a standard light/dark room at a constant temperature ( $22 \pm 1^\circ\text{C}$ ) and humidity. Ovariectomies (OVX) was performed through dorsal incision. Two weeks after the operation, myocardial infarction was induced by ligating the left anterior descending artery. Three OVX groups (10 rats in each group) were studied: (1) without induced myocardial infarction (MI), but supplemented with estrogen; (2) induced MI, but without supplemented with estrogen; and (3) MI + supplemented group. Supplemented animals received  $17\beta$ -estradiol from D1.  $17\beta$ -estradiol was supplemented by Depo-estradiol (estradiol cypionate injection USP; Up-john, Kalamazoo, MI), which was diluted into 10  $\mu\text{g/mL}$  with cotton-seed oil and was administered intramuscularly once per 2 week. A single intramuscular injection of Depo-estradiol is sufficient to maintain a steady serum concentration of estradiol for at least 19 days (16). The drugs were used for 4 weeks starting on the day of randomization. Sham operation served as controls. The study duration was designed to be 4 weeks because the majority of the myocardial remodeling process in the rat (70-80%) is complete within 3 weeks (17).

#### *Experimental myocardial infarction*

To create the model, rats were anesthetized with ketamine (90 mg/kg) intraperitoneally. After adequate anesthesia they were intubated with a 14-gauge polyethylene catheter and ventilated with room air using a small animal ventilator (model 683, Harvard Apparatus, Boston, MA). The heart was exposed via a left-sided thoracotomy, and the anterior descending artery was ligated using a 5-0 silk between

the pulmonary outflow tract and the left atrium. The muscle and skin were closed in layers. Sham rats underwent the same procedure except the suture passed under the coronary artery and then removed. Mortality rate of infarcted rats after the operation was 50%.

#### *Hemodynamics and Infarct size measurements*

After the last arterial pressure measurement, the rats were anaesthetized with thiopental sodium (50 mg/kg, ip). A left thoracotomy was performed through the intercostal space. The LV apex was immediately punctured using a 25-gauge fluid-filled needle attached to a pressure transducer. Left ventricular end-systole and end-diastole pressure were measured without damped wave forms. Next, the heart was rapidly excised and suspended for retrograde perfusion with a Langendorff apparatus. At completion of the electrophysiological tests, a 1 to 1.5 mm coronal section, taken from the equator of the heart, was fixed in 10% formalin and embedded in paraffin for determination of infarct size. Each section was stained with hemotoxylin and eosin, and trichrome. The areas of scar and nonscar regions were measured the tracings by computerized planimetry (Image Pro Plus, Media Cybernetics, Silver Spring, MD) at the same mid-papillary slice of each heart. The infarct size was determined according to method of Pfeffer et al (18): the lengths of scar for the endocardial and epicardial surfaces were summed as endocardial and epicardial circumferences. The rest of the tissue were divided in right ventricle, septum, infarcted, or adjacent noninfarcted left ventricular wall. The samples were rapidly frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for ET-1 levels.

### ***Perfusion of isolated hearts***

Each heart was perfused with a modified Tyrode's solution containing (in mM): NaCl 117.0, NaHCO<sub>3</sub> 23.0, KCl 4.6, NaH<sub>2</sub>PO<sub>4</sub> 0.8, MgCl<sub>2</sub> 1.0, CaCl<sub>2</sub> 2.0, and glucose 5.5, equilibrated at 37°C and oxygenated with a 95% O<sub>2</sub>-5% CO<sub>2</sub> gas mixture. The perfusion medium and the chamber containing the isolated heart were maintained at a constant temperature of 37°C. The aortic pressure was 100 mm Hg and connect to a high fidelity microtip catheter (Gould Inc.) on the Gould physiological recorder. Epicardial electrograms were recorded by an atraumatic unipolar electrode, placed on the anterior left ventricular wall 2 mm below the circumflex artery. The reference electrode was placed in the perfusion bath.

### ***Spontaneous and Induced arrhythmias***

After isolation, the hearts was observed for 20 minutes to allow stabilization of hemodynamics. During the period, spontaneous arrhythmias was recorded. Induced arrhythmias was effected using an electrical Bloom stimulator. Stimulation intensity was twice the threshold, and stimulus length was 5 msec. The protocol for pacing was modified from that of Nguyen et al (19). The heart was stimulated with a train of eight stimuli at a cycle length of 100 ms (S<sub>1</sub>), followed by one to three extrastimuli (S<sub>2</sub>, S<sub>3</sub>, and S<sub>4</sub>) at shorter coupling intervals. The end point of ventricular pacing was induction of ventricular tachyarrhythmia consisting of at least 6 consecutive ventricular extrastimulus beats. A preparation was considered non-inducible when pacing produced either no VPC or only self-terminating salvos of <6 beats. A ventricular tachyarrhythmias including ventricular tachycardia and ventricular fibrillation were considered nonsustained

when it lasted ≤15 beats and sustained when it lasted >15 beats. An arrhythmia scoring system was used as previously described (19).

### ***Immunohistochemical analysis of ET-1***

In order to investigate the spatial distribution of ET-1, immunohistochemical staining was performed on LV muscle from border zone (0 to 2 mm outside the infarct) and non-ischemic areas (>2 mm outside the infarct). Hearts were snap-frozen in liquid nitrogen, embedded in OCT compound (Tissue-Tek), and cryosections were performed at a thickness of 7µM. The slides containing the sectioned tissues were rehydrated in 0.01% sodium bicarbonate at pH 7.4. Sections were blocked with 0.1 mmole/L L-lysine in PBS containing 0.1% triton X-100 for 45 minutes. Tissues were incubated with a rabbit polyclonal anti-ET-1 antibody at dilution 1:200 in 0.5% BSA in PBS overnight at 37°C. Immunostaining with ET-1 antibodies was performed using a standard immunoperoxidase technique (N-Histofine Simple Stain MAX PO kit, Nichirei Co., Tokyo, Japan). The antibody used had been tested for specificity in the rat. Negative controls were performed by omitting the primary antibody. Because of the wide variability of structural composition of border zone regions which resulted in intercellular connection ranging from total disruption in fully scarred regions to negligible alterations with normal appearing myocytes, we selected samples for analysis that were composed of cardiomyocytes separated by diffuse interstitial fibrosis.

### ***Plasma and tissue levels of ET-1 and plasma estradiol levels.***

Because of a local release of ET-1 at the border zone, blood samples from the aortic root and the tissue from the border zone and

remote interventricular zone were obtained for measurements of systemic and local ET-1 levels at the end of the study. Plasma ET-1 concentration was measured by collecting 4 ml of blood in test tubes containing 2% ethylenediaminetetraacetic acid (80  $\mu$ l/ml of blood). Blood samples were immediately centrifuged at 3,000g for 10 minutes, and the plasmas were stored at  $-70^{\circ}$ C until further analysis. The myocardiums were homogenized with a polytron homogenizer for 60 seconds in 10 vol. Of 1 mol/l acetic acid containing 10  $\mu$ g/ml pepstatin and immediately boiled for 10 minutes at  $4^{\circ}$ C. ET-1 was measured by immunoassay (R&D System Inc, Minneapolis, MN). Plasma (1 ml) was acidified with 3 ml of 4% acetic acid and ET-1 was extracted with a Sep-pak C-18 cartridge. The detection limit was 1 pg/ml for ET-1. Intra-assay and inter-assay coefficients of variation was 4.5% and 6.6%, respectively.  $17\text{-}\beta$  estradiol concentrations were quantified by enzyme-linked immunoassay (Diagnostic Products Corporation, Los Angeles, CA) at the end of the study. The detection limit was 10 pg/ml for estradiol.

### *Cell isolation*

Because cardiac hypertrophy is a combination of reactive fibrosis and myocyte hypertrophy, we measured cardiomyocyte sizes from the border zone besides using myocardial weight to avoid the interference of non-myocytes on post-infarction hypertrophy. Since the infarct size measurement procedure does not permit quantitation of cardiomyocyte sizes, additional groups of rats ( $n = 5$  in each group) were infarcted using the same procedures and used for measurement of cell sizes at the end of the study. Myocytes were enzymatically isolated according to previously described techniques (20). Briefly, the rats were

heparinized; and heart excised and perfused at a constant flow of 8 ml/min by a modified Langendorff technique at  $37^{\circ}$ C with a nominally  $\text{Ca}^{2+}$ -free, oxygenated Tyrode solution (pH 7.4) containing (in mM): NaCl 137, KCl 5.4,  $\text{MgCl}_2$  1.1, dextrose 11, HEPES 10. After 5 min of equilibration, the perfusion was changed to the same solution containing 0.34 mg/ml collagenase (type II; Sigma Chemical Co., St, Louis, Mo., USA) and 0.08 mg/ml protease (type XIV, Sigma). After 10 to 15 min of digestion, the residual enzyme-containing solution was cleaned by 5-min perfusion with 0.2 mM  $\text{Ca}^{2+}$  Tyrode solution. Then, the heart was removed from the cannula, the undigested infarct area was removed, and the LV, including border zone (0-2 mm outside the infarct) and interventricular septum, was mechanically dispersed. Random high-power fields of the rod-like relaxed myocytes with clear striations were selected under confocal microscopy (LSM-410 Invert, Zeiss) to eliminate selection bias. At least 20 cells from each section were selected for measurement of cell length, width and area, and the mean value was used as the individual value for each section. Although it is impossible to isolate myocytes from hearts subjected to confocal study, infarct size should be considered to be similar within various groups because animals were randomly assigned to confocal or cellular study. In the sham-operated group, cell width and length were measured from the ventricular free wall and left ventricular septum for comparisons.

### *Statistical Analysis*

Results were presented as mean  $\pm$  SD. According to the size of myocardial infarction, rats were divided into 3 groups: small MI (<10%); moderate MI (10-35%) and large MI (>35%). With respect to

clinical importance, only rats with large MI were selected for detailed investigation. Differences among the groups of rats were tested by a one-way ANOVA. Subsequently analysis for significant differences between the two groups were performed with a multiple comparison test (Scheffe's method). Electrophysiological data (scoring of programmed electrical stimulation-induced arrhythmias) were compared by a Kruskal-Wallis test followed by a Mann-Whitney test. The significant level was assumed at value of  $P < 0.05$ .

#### 四、Results

Differences in mortality between placebo and treated groups were not found throughout the study.

##### *Hemodynamics*

Although the LV end-diastolic pressure was not affected by estrogen administration, myocardial infarction induced marked increase of the LV end-diastolic pressure compared with the sham-operated group.

##### *Morphometric studies*

Body weights were unchanged by infarction or treatment. The infarcted rats without supplemented estradiol had an increase in left ventricular weight/BW ratio, right ventricular weight/BW ratio, and lung weight/BW ratio compared with sham-operated rats (Table 1). Four weeks after MI, the infarcted area of the LV was very thin and was totally replaced by fully differentiated scar tissue.

To characterize the cardiac hypertrophy on a cellular level, we isolated cardiomyocytes from different treated groups. The cells isolated from the border zone in the control group were obviously larger than the cells from the same area of sham-operated hearts. After infarction, cell size increased

by 45% ( $P < 0.0001$ ) at the border zone. This was attenuated by estrogen administration to 6%. The cell width and length of the estrogen-treated myocytes were significantly smaller than vehicles (Table 2). The finding was compatible with the notion that post-infarction angina is a regional process that progressed from the border zone to involve the entire LV. No regional differences of cardiomyocyte sizes were found in sham-operated rats.

##### *Electrophysiological stimulation*

Arrhythmia scores in sham-operated rats were very low (0). In contrast, ventricular tachyarrhythmias consisting of ventricular tachycardia and ventricular fibrillation were inducible by programmed stimulation in rats with MI ( $3.1 \pm 1.2$ ). Estrogen treatment decreased the inducibility of ventricular tachyarrhythmias compared with those in the vehicle ( $1.3 \pm 1.0$ ,  $P < 0.0001$ ).

##### *Circulating and myocardial ET-1 levels*

Circulating ET-1 levels remained similar in infarcted rats among the groups. To investigate the possible role of cardiac ET-1 synthesis to the reduction of plasma ET-1 levels, we determined the ventricular ET-1 levels. Expression was region dependent with a significant increase at the border zone compared with that in the interventricular septum after large infarctions (Table 3). LV ET-1 levels were significantly lower in estrogen-treated rats than in the vehicle-treated rats.

##### *Immunohistochemical analyses*

Immunohistochemical analysis of the infarcted myocardium revealed the presence of ET-1 immunoreactivity in the myocardial tissue. Figure shows slightly stronger ET-1 in the nonischemic myocardium of control rats