Atrial Natriuretic Peptide, Heat Shock Protein 70 and c-FOS Modulate Estrogen Protective Effect on Cardiac Performance in Female Rats Subjected to Immobilization Stress

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Abstract

Several international clinical trials have reported controversial effects of estrogen replacement therapy on the cardiovascular system. The aim of this study was to investigate the effects of estrogen on myocardial performance under conditions of simulated emotional stress in female rats and to elucidate some of its intracellular mediators in the myocardium.

Material and Methods: 64 female albino rats were included in this study and classified into four main groups; each group included 16 rats.

Group 1 (Control group), group 2 immobilization stress group (IMO), group 3 (IMO + ovariectomy), group 4 (IMO + Ovariectomy + estrogen supplementation 10 µg/100gm, once daily by intramuscular injection for 2 weeks). At the end of the experimental protocol, evaluation of cardiac performance in isolated hearts and measurement of cardiac tissue gene expression of c-FOS, HSP 70 and ANP m-RNAs was conducted.

Results: Immobilization stress (IMO) group showed significant increase in systolic blood pressure (SBP) and diastolic blood pressure (DBP). The animals also showed a significant increase in gene expression of c-FOS (functional marker of cellular activation) and heat shock protein (cardioprotective substance, HSP70) and significant decrease in contractility index in comparison to control group.

Ovariectomized immobilized group (OVE + IMO group 3) showed significant increase in SBP, DBP, c-FOS expression and significant decrease in atrial natriuretic peptide (ANP) and HSP70 expression in comparison to IMO group.

Administration of estrogen to OVE rats followed by immobilization stress caused a significant decrease in SBP, DBP, c-FOS expression and a significant increase in ANP and HSP70 expression in comparison to IMO + OVE group.

Conclusion: Estrogen up-regulation of expression of ANP and HSP 70 and down-regulation of c-FOS in the myocardium can confer a protective effect on myocardial performance in states of immobilization stress.


Introduction

THERE is growing evidence that psychosocial stress can influence the natural history of cardiovascular disease [1]. Several studies indicated that psychosocial factors both contribute to the development of coronary artery disease and increase risk of cardiac dysfunction and the likelihood of cardiac events in susceptible patients with established disease [2,3].

Men and women may differ in their cardiovascular responses to mental stress [4]. Bairey Merz, et al. [5] found that women had greater blood pressure, heart rate and rate-pressure product responses to mental stress than men. They also reported that women of postmenopausal age have greater cardiovascular responses to stress than men or premenopausal women. However, Strike and Stoptoe [6] conducted a review of the literature on the subject and found that there is insufficient information to draw firm conclusions about the prevalence and significance of mental stress induced myocardial pathology in women and the role that estrogen can play under mental stress conditions.

The role that estrogen replacement therapy might play on different body systems in postmenopausal women under different conditions has received a lot of attention.

The Women’s Health Initiative (WHI) in healthy postmenopausal women without coronary heart disease demonstrated that hormone replacement therapy was associated with an initial increased risk of cardiovascular disease [7].
Similarly, the Women’s Estrogen for Stroke Trial (WEST) found that estrogen increased the risk for either fatal stroke or more severe neurological impairment after stroke [8].

Alternatively, Wakatsuki, et al. [9] have shown previously that postmenopausal estrogen replacement therapy (ERT) may reduce the risk of atherosclerosis by the combined effects of reducing plasma concentrations of low-density lipoprotein (LDL), increasing high-density lipoprotein (HDL) and improving endothelium-dependent vasodilatation [10].

The aim of the present study was to investigate the possible effects of estrogen on myocardial performance under conditions of emotional stress. This aim was achieved through evaluation of myocardial performance in isolated hearts of female rats subjected to immobilization stress with or without prior ovariectomy or ovariectomy with estrogen supplementation. The study also included measurement of myocardial expression of c-FOS which is a marker of cellular stress and heat shock protein 70 (HSP 70) as well as atrial natruritic peptide (ANP) which are considered as important mediators of myocardial protection against stress induced injury.

Material and Methods

Experimental animals:

Sixty four female albino rats (150-180gm) were included in the present study but only 60 animals survived the experimental protocol till the end. All rats were housed in wire mesh cages at room temperature, with 12:12-h light-dark cycles and maintained on rat chow and tap water for the duration of the study protocol which lasted for two weeks. The research was approved by the ethical committee of Kasr Al-Aini Faculty of Medicine, Cairo University.

The animals were divided randomly into four main groups:

- **Group 1**: Control group (C): (n=16 rats).
- **Group 2**: Immobilization group (IMO): The animals were subjected to an immobilization stress protocol for thirty minutes prior to any measurements (n=16).
- **Group 3**: The animals were subjected to bilateral ovariectomy (OVE) followed two weeks later by the immobilization stress protocol (IMO + OVE) (n=14).
- **Group 4**: The animals were subjected to ovariectomy and received estrogen (E) supplementation followed two weeks later by the immobilization protocol (IMO + OVE + E) (n=14 rats).

Estrogen was administrated by intramuscular injection (i.m) 10\(\mu\)g/100gm body weights, once daily for 2 weeks [11].

The estrogen used was bought as estrogen ampoules, each 1ml ampoule contains: Oestradiol benzoate 5mg (Folone) in oily solution produced by MISR Co. For Pharma. Ind. S.A.E.

Ovariectomy technique:

Animals in group (3) and (4) underwent bilateral ovariectomy under light ether anesthesia. A longitudinal incision was done in the midline of the animal back just caudal to 13 th rib; the incision was opened to expose each ovary. A small puncture was done over the site of the ovary, which can usually be seen embedded in a pad of fat in the abdomen. Care was taken in order not to rupture the capsule of the ovary. The ovaries were removed by a cut with a scissor at fallopian tube. The wound was sutured and covered by a local antibiotic [11]. A sham ovariectomy consisting of a skin incision and suture under anesthesia was performed for the other two groups.

Immobilization stress protocol:

Immobilization stress (IMO) in the rat provides an excellent animal model of emotional stress, which activates the hypothalamic-pituitary-adrenocortical system and the sympatho-adrenal system [12].

Two weeks after ovariectomy, rats of group 2, 3 & 4 were exposed to immobilization stress (IMO) for 30 minutes by fixation to a wooden board [13,14]. The animals showed a defection and sometimes urination immediately after fixation to the wooden board.

Measurements:

After finishing the immobilization protocol, each group was subdivided into 2 subgroups; (a) and (b). The hearts of the animals in subgroup (a) were isolated to measure myocardial performance using a langendorf apparatus while the hearts of subgroups (b) were used to measure gene expression of c-FOS, HSP70 and ANP mRNA by RT-PCR (4 animals died).

I- Subgroups (1 a, 2 a) included 8 rats while (3 a, 4 a) included 7 rats.

II- Subgroups (1 b, 2 b) included 8 rats (3 b, 4 b) included 7 rats.
Measurement of left ventricular performance using isolated heart preparations:

Rats of Subgroups 1, 2, 3, 4 were anesthetized and their hearts isolated for measurement of myocardial performance using a Langendorff apparatus as previously described [15]. Briefly, a left thoracotomy was performed to expose the heart. Exposed hearts were then immediately excised through a pericardial incision at the base of the heart. Hearts were then quickly placed in ice cold modified Krebs-Henseleit solution, transferred and attached to modified Langendorff apparatus by the aortic root through the perfusion cannula.

The time between extraction of the hearts and their attachment to the lagendorff apparatus did not exceed two minutes. Hearts were then perfused retrogradely with non-recirculating modified oxygenated Krebs-Henseleit solution of the following concentration in (mM) 118 NaCl, 25 NaHCO3, 1.2 KH2PO4, 4.7 KCl, 1.2 MgSO4, 2 CaCl2 and 10 dextrose in 1000ml distilled water. The perfusate was maintained at 38 °C and the hearts were also maintained at 38 °C using a water reservoir surrounding the hearts in which the open end was covered to maintain temperature and humidity.

To record intra-ventricular pressures, a saline filled latex balloon connected to a catheter was inserted into the left ventricle. The catheter was then connected to a pressure transducer (Gold statum). Pressure changes were then analyzed and displayed on an electronic polygraph (NEC-San- ei, 2238). The intraventricular balloon was then filled with saline to adjust the baseline end diastolic pressure (EDP) to 10mmHg. Hearts were then left to stabilize for 30 minutes.

Parameters of myocardial performance measured:

The following parameters were then measured: Left ventricular systolic pressure (SBP), diastolic pressure (DBP), left ventricular developed pressure (LVDP) designated as the difference between systolic and diastolic left ventricular pressures and heart rate (HR). The peak rate of maximum left ventricular pressure rise (dP/dT) was also measured as an index of contractility.

Measurements of c-FOS, HSP 70 and ANP mRNA gene expression:

Rats of Subgroups 1, 2, 3, 4 were killed by a blow on the head and their hearts were rapidly removed (within 1 min after decapitation), frozen using powdered dry ice and stored at –80 °C in RNA lysis buffer that contain guanidine thiocyanate and B-mercapto-ethanol for RA extraction, until sectioned for measurement of gene expression of: c-Fos m RNA, Atrial naturetic peptide m RNA and Heat shock protein m RNA (HSP 70) by RT-PCR.

For the detection of c-FOS, ANP & HSP70 gene expression, the following parameters were performed: RNA extraction, RNA reversion & transcription into CDNA, DNA amplification by PCR and Detection of the Amplified DNA.

Briefly, about 10mg of heart tissue was weighed and then homogenized in lysis buffer, then centrifuged at 10,000 RPM for 10 minutes. The supernatant of tissue homogenate was subjected to RNA extraction by using SV total RNA extraction system kit (promega, Madison WI, USA) according to manufactures recommendation.

Single strand CDNA was prepared from RNA using oligodt primer as follows:

Total RNA (20ug) was heated at 95 °C for 5 mm with 50pmol primer (oligodt) before adding 2lil of 5XRT buffer (50mM tris Hcl, pH 8.3, 10mM MgCl2, 75mM kcl), 68 units of RNase inhibit 2.5mM dinucleotides triphosphate dNTPs (promega) and 10 units of murine leukemia virus reverse transcriptase (pharmacia) in a final volume of 20Ftl after incubation for 2 hours.

The oligonucleotide primers sequence of studied genes are as follows:

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP</td>
<td>Forward primer: ATACAGTGCGGTGTTCCAACA reverse primer: CGAGAGCACCTCCATCTCTCT</td>
</tr>
<tr>
<td>HSP 70</td>
<td>Forward primer: TCTAACACGCTGGCTGAGAA reverse primer: CACCCTGAGAGCCAGAAAAG</td>
</tr>
<tr>
<td>c-FOS</td>
<td>Forward primer: GTGAGACCATGTCAGGAC reverse primer: TTGATCTGTCTCCGCTT G</td>
</tr>
</tbody>
</table>

The PCR reaction was carried as follows: cDNA was added to 50pmol each of forward and reverse primer, 0.2mM ci, NTPs 2 unit Taq polymerase (promega) and 1 OX. reaction buffer (promega, Madison). The PCR was for 40 cycles including denaturation (94°C for 1min) annealing (60°C for 1min) and elongation (72°C for 1min) followed by final extension step at 72°C for 10min.
Agarose gel electrophoresis: Amplified PCR products were electrophoresed on 2% agarose gel with size marker and were UV visualized by ethidium bromide staining. The UV gel was photographed and the densitometry was analysed using Biometra gel documentation system (USA).

Statistical analysis: The data was encoded and entered using the statistical package SPSS Version 15. The data was summarized using mean, standard deviation and range for quantitative variables. Comparison between studied groups was done using ANOVA (analysis of variance) and multiple comparisons Post Hoc Test for normally distributed quantitative variables. The p-value ≤0.05 were considered statistically significant.

Results

Myocardial performance parameters: Our results show a significant increase in SBP & DBP (p≤0.05) of immobilized group rats and a significant reduction in their contractility index (dp/dt) when compared to their corresponding values in control group (p≤0.05).

In group 3 subjected to ovariectomy prior to immobilization stress, there was a significant increase in SBP & DBP when compared to group 2 immobilization group (p≤0.05).

Estrogen supplementation after ovariectomy in Group 4 (OVE+E+IMO) resulted in a significant increase in SBP & DBP and HR (p≤0.05) and no significant change in LVDP and dp/dt (p≥0.05) when compared to group 3 (OVE+IMO) (Table 1).

Results of gene expressions of c-FOS, ANP and HSP 70:

Our results show a significant increase in c-FOS & HSP70 (p≤0.05) but insignificant increase in ANP in IMO group 2 when compared to corresponding values in control group (p≥0.05). (c-FOS=7.76±1.11 versus 5.56±0.81μg/dl, HSP=3.52±0.46 versus 2.26±0.73μg/dl, ANP=2.1±0.48 versus 1.73±0.55μg/dl).

In animals subjected to ovariectomy prior to the immobilization stress, there was a significant increase in c-FOS expression (p≤0.05) and a significant reduction in expression of ANP and HSP 70 (p≤0.05) when compared to corresponding values in immobilization group. (c-FOS=18.66±1.51 versus 7.76±1.1μg/dl, HSP=1.76±0.39 versus 3.52±0.46μg/dl, ANP=0.45±0.26 versus 2.1±0.48μg/dl).

In animals receiving estrogen supplementation after ovariectomy (OVE+E+IMO) in group (4), there was a significant decrease in c-FOS expression and a significant increase in HSP 70 and ANP expression when compared to corresponding values in ovariectomy group 3. (p≤0.05) (c-FOS=11.64±1 versus 18.66±1.51μg/dl, HSP=2.64±0.57 versus 1.76±0.39μg/dl, ANP=1.83±0.79 versus 0.45±0.26μg/dl). However, c-FOS expression in group 4 was still significantly higher and HSP was still significantly lower than corresponding values in immobilization group 2 (c-FOS=11.64±1 versus 7.76±1.1μg/dl and HSP=2.64±0.57 versus 3.52±0.46μg/dl) (p≤0.05). However, ANP expression in group 4 reached almost the same level encountered in group 2 with no statistically significant difference (p≥0.05) Figs. (1-4).

Table (1): Effect of immobilization (IMO), ovariectomy (OVE) and (IMO) and immobilization in animals receiving intramuscular (i.m.) estrogen supplementation (E) at a dose (10 μg/100gm) after OVE on myocardial performance parameters of isolated hearts of studied animals.

<table>
<thead>
<tr>
<th>Group (1)</th>
<th>Group (2) IMO</th>
<th>Group (3) OVE+IMO</th>
<th>Group (4) OVE+E+IMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=8</td>
<td>n=8</td>
<td>n=7</td>
<td>n=7</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>95.5±7.32</td>
<td>126.38±19.85</td>
<td>159.43±25.96</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>71.75±8.31</td>
<td>105.13±23.81</td>
<td>143.28±24.47</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>23.25±3.88</td>
<td>21.25±6.88</td>
<td>16.14±5.78</td>
</tr>
<tr>
<td>Heart rate (beat/min)</td>
<td>69.25±13.14</td>
<td>82.63±23.84</td>
<td>110.85±51.1</td>
</tr>
<tr>
<td>Contractility dp/dt (mmHg/sec)</td>
<td>550±193.53</td>
<td>213.75±55.53</td>
<td>215.57±74.83</td>
</tr>
</tbody>
</table>

Results are mean±SD.
IMO: Immobilization stress. LVDP: Left ventricular developed pressure.
SBP: Systolic blood pressure. HR : Heart rate.
DBP: Diastolic blood pressure. dp/dt : Contractility index.
E = Estrogen (10μg/100g body weight) i.m.
a: Significant change when compared to control group 1. p≤0.05
b: Significant change when compared to (IMO) group 2. p≤0.05
c: Significant change when compared to (OVE+IMO) group 3. p≤0.05
Results are mean±SD. Columns are arranged from left to right in each box to represent control, immobilization, ovariectomy+immobilization and ovariectomy + estrogen + immobilization groups sequentially.

ANP = Atrial naturitic peptide, HSP = Heat shock protein, IMO = Immobilization stress, OVE = Ovariectomy.
E = Estrogen (10ug/100g body weight) i.m.

a: Significant change when compared to control group 1. p<0.05
b: Significant change when compared to (IMO) group 2. p<0.05
c: Significant change when compared to (OVE+IMO) group 3. p<0.05

Fig. (4): Diagrammatic representation for gene expression of c-FOS, ANP and HSP70 in myocardial tissue homogenates of the four groups studied.
Discussion

The results of our work showed that immobilization stress resulted in a significant increase in systolic blood pressure, diastolic blood pressure and significant decrease in contractility index.

Immobilization in the rat has been used as a model of emotional stress that activates the hypothalamic-pituitary-adrenocortical system and the sympatho-adrenal system [12]. The haemodynamic response to mental stress typically involves increased heart rate and blood pressure, the latter being sustained by raised systemic vascular resistance, cardiac output, or a combination of the two. A related factor is catecholamine release. Venous adrenaline and nor-adrenaline increased rapidly with mental stress and to a larger degree in men than in women [16,17].

Ueyama, et al. [18] demonstrated that IMO stress temporarily impaired left ventricular function and resulted in an increase in heart rate and blood pressure, an effect which was blunted by estrogen supplementation. Our results did not show a significant increase in heart rate after immobilization when compared to controls, however, we acknowledge that our sample size was limited and possibly a larger number of measurements might have elucidated this effect. It is also possible that the timing involved between the end of the stress period and isolation of the hearts for measurements had an effect on the results obtained.

Kvetnansky, et al. [19] suggested that the elevated blood pressure and heart rate during stress help the body handle the emergency and restore homeostasis. However, continuation of these effects following cessation of the stress is implicated with many stress-related disorders.

High levels of catecholamine can damage cardiac tissue and diminish left ventricular function because of Ca2+ overload, coronary occlusion, free radical formation and myocardial ischemia [20].

Our results also showed an increased expression of c-FOS and of heat shock protein 70 in immobilized animals of group (2) when compared to control group animals.

c-Fos mRNA is a functional marker of cellular activation induced in cellular stress and its induction is associated with cell death by apoptosis or necrosis [21] while heat shock protein 70 (HSP 70) and atrial naturetic peptides (ANP) are cardio protective substances [22,23].

HSPs maintain cellular homeostasis and survival in response to stressful cellular conditions. Involvement of HSP families in many pathological conditions has been also extensively studied [24]. The increased levels of HSP following hypoxia were associated with the recovery of contractile function in isolated heart preparations probably via an energy metabolism [25].

ANP is released by atrial myocytes in response to elevated blood pressure. ANP acts to reduce the water, sodium and adipose loads on the circulatory system, thereby reducing blood pressure to normal levels [26].

The present results coincide with that of Ueyama, et al. [22] who proved an induction of c-FOS in the hearts of immobilized rats which was prevented by pretreatment with combined blockade of $\alpha$ and $\beta$ adreno-receptors suggesting that enhanced sympatho-adrenal outflow is involved in c-FOS up-regulation.

Our results showed an increase of SBP and DBP in immobilized ovariectomized animals in group (3) when compared to immobilized group (2). Group (4) animals which received estrogen supplementation after ovariectomy showed a drop of blood pressure and heart rate when compared to group (3).

Ovariectomized immobilized animals (group 3) also showed a significant increase of c-fos mRNA and significant decrease of ANP mRNA and HSP70 mRNA when compared to group (2) animals. Supplementation with estrogen in group 4 prevented the marked increase of cFOS and also resulted in a significant increase of HSP70 mRNA & ANP mRNA when compared to group (3).

McCubbin, et al. [27] and Komesaroff, et al. [28] reported that estrogen can reduce blood pressure and heart rate in postmenopausal women subjected to mental stress and suggested that this effect could be partly modulated through a reduction of sympathetic activity.

Estrogen might affect sympatho-adrenal system through a central mechanism as Ueyama, et al. [29] found that immobilization in experimental animals increases cFOS mRNA expression in central sympathetic neurons that also express immunoreactive estrogen receptors. Because ER $\alpha$ and ER $\beta$ are expressed in the cardiac cells, estrogen can directly act to reduce the reactivity to catecholamines in the heart. Administration of estrogen reduced isoproterenol-induced tachycardia and the incidence
of ischemia/reperfusion-induced arrhythmia and cAMP production in rat hearts [30].

It is thus possible that ovariectomy would result in an increase in sympathetic-adrenal functions leading to an increase in both systolic and diastolic pressure and can lead to an increase in expression of c-FOS in cardiac myocytes while estrogen supplementation would reduce these findings as encountered in group 4 animals.

The upregulation of ANP and HSP in the myocardium by chronic estrogen treatment was also reported by VanEickels [31] and the up-regulation of ANP in the heart is mediated via the ER α-mediated pathway [32].

ANP can exert the cardioprotective actions not only as a circulating hormone but as local autocrine and/or paracrine factors [33]. ANP can decrease the stress-induced increase of an afterload by natriuresis, inhibition of renin–angiotensin–aldosterone system and vasodilation. ANP also counteracts the stimulated Ca^{2+} influx into cardiomyocytes by a cGMP mediated mechanism [34].

Voss, et al. [35] also found that ovariectomy reduced the level of HSP70 in female hearts and this was prevented by estrogen supplementation. They reported that cardiac HSP70 levels in females are 2-fold higher than that in males [38].

We conclude that estrogen plays a protective effect on the myocardium under conditions of emotional stress partly through reduction of sympathetic-adrenal activity and reducing their effect on the heart. This protective role is partly mediated by a reduction of c-FOS expression and increased expression of atrial natriuritic peptide and heat shock protein 70 in myocardial tissues. However, more research is required to clarify the role played by gender and sex hormones in regulation of myocardial and vascular responses to emotional stress.

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172


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