Effects of Estrogen Replacement on Infarct Size, Cardiac Remodeling, and the Endothelin System After Myocardial Infarction in Ovariectomized Rats

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Background—Estrogen may increase the long-term survival of women who have suffered from a myocardial infarction (MI). We examined the acute and chronic influence of estrogen on MI in the rat left coronary artery ligation model.

Methods and Results—Female Sprague-Dawley rats (10 to 12 weeks, n = 93), divided into 3 groups (rats with intact ovaries, ovariectomized rats administered 17β-estradiol [17β-E2] replacement, and ovariectomized rats administered placebo 2 weeks before MI), were randomized to left coronary artery ligation (n = 66) or sham-operated (n = 27) groups.

Ten to 11 weeks after MI, rats were randomly assigned to either (1) assessment of left ventricular (LV) function and morphometric analysis or (2) measurement of cardiopulmonary mRNA expression of preproendothelin-1 and endothelin A and B receptors. Acutely, estrogen was associated with a trend toward increased mortality. Infarct size was increased in the 17β-E2 group compared with the placebo group (42 ± 6% versus 26 ± 3%, respectively; P = 0.01). Chronically, wall tension was normalized through a reduction in LV cavity size with estrogen treatment (419 ± 61 mm Hg/mm for 17β-E2 versus 946 ± 93 mm Hg/mm for placebo, P = 0.039). In the LV, there was a 2.5-fold increase in endothelin B mRNA expression after MI in placebo-treated rats (P = 0.004 versus sham-operated rats) that was prevented in the 17β-E2 group (P = NS versus sham-operated rats).

Conclusions—These results suggest that estrogen is detrimental at the time of MI or early post-MI period, resulting in an increased size of infarct or infarct expansion, but chronically, it can normalize wall tension and inhibit LV dilatation, which may in turn lead to increased long-term survival. Regulation of the endothelin system, particularly the expression of the endothelin B receptor, may contribute to these estrogenic effects.

Key Words: hormones • myocardial infarction • endothelin
Female Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) aged 10 to 12 weeks (n=93, average weight 252 g) were randomized to 3 groups: (1) rats with intact ovaries, (2) ovariectomized rats administered 17β-estradiol (17β-E₂) replacement, and (3) ovariectomized rats administered placebo. Treatments were administered by means of a single subcutaneous pellet inserted into the dorsal neck region under anesthesia. To achieve typical estradiol levels found in midcycle (~50 pg/mL), a 90-day release pellet containing 1.7 mg 17β-E₂ (Innovative Research of America) was used.

**Induction of MI**

Two weeks after hormone replacement, animals from the 3 groups were randomized to the LCA ligation group (n=66) to induce MI or to the sham-operated group (n=27), as described previously. Acute mortality was defined as death within 24 hours of the operation.

**Assessment of LV Function in Isolated Heart Preparation**

Ten to 11 weeks after surgery, a subgroup of rats (n=30) was randomized to undergo hemodynamic analysis with use of the Langendorff perfusion apparatus, as described, for assessment of left ventricular (LV) function.

**Cardiac Morphometry**

After the hemodynamic measurements were completed, hearts were perfusion-fixed and sectioned as described previously. Morphometric analysis was performed on each section by using a quantitative digital analysis system (Simple, C-Imaging Systems). Septal (noninfarcted) and lateral (infarcted) wall thickness was measured and averaged from 3 equidistant points on an axis that cut the endocardial surface at 90°. LV endocardium was traced, and LV cavity area, as well as the LV epicardial surface, was assessed by planimetry.

**Relative Infarct Size**

The relative infarct size was determined according to the method of Pfeffer et al. Infarct size was defined as the ratio of the lengths of scar and of surface circumferences.

**Peak Wall Tension**

With the use of peak systolic pressure values obtained from the Langendorff preparation, the average peak wall tension was calculated for each heart by using the following formula: peak LV systolic pressure (mm Hg) × LV cavity area (mm²) / 2 × septal thickness (non-infarcted wall, mm).

**RNA Isolation**

RNA was extracted from LV and lung tissue of a second group of rats (n=63) by use of an RNeasy Mini Kit (QIAGEN Inc).

**Assessment of Cardiovascular Prepro-ET-1 mRNA Expression**

Prepro-ET-1 mRNA levels in the LV were measured with a ribonuclease protection assay kit (HybSpeed RPA, Ambion Inc) as described previously.

**Assessment of Cardiovascular ETₐ and ETₜ Receptor mRNA Expression**

ET receptor mRNA levels were quantified by competitive reverse transcription–polymerase chain reaction (PCR), as described previously.

**Immunohistochemical and Immunofluorescence Analyses**

Fixed paraffin-embedded serial sections (4 to 6 μm) from 12 hearts (n=3 per group: sham-operated and MI groups receiving placebo or 17β-E₂) were deparaffinized and incubated for 30 minutes in 0.1% saponin/PBS/1% BSA to permeabilize cellular membranes. Primary protein G–purified sheep anti-ETₐ (Research Diagnostics Inc) antibodies were used at 1:100. After an overnight incubation and stringent washing, rabbit anti-sheep IgG (1:500; Sigma Chemical Co) was added to the sections, followed by use of the biotin/avidin detection system (Vectastain ABC kit, Vector Laboratories). To visualize the ETₐ receptors, NovaRED (Vector Laboratories) was used as a chromagen. For immunohistochemistry, the sections were subsequently counterstained with hematoxylin, dehydrated, cleared, and mounted. Immunofluorescent staining was performed with secondary FITC-labeled goat anti-rabbit IgG (Sigma). Cell nuclei were counterstained with ethidium bromide at 1 μg/mL for 2 minutes. The sections were examined with use of a Bio-Rad MRC-600 laser-scanning confocal imaging system equipped with Bio-Rad COMOS operating software. Scar tissue was delineated by staining with picrosirius red.

**Measurement of Serum 17β-E₂ and Plasma ET-1**

Blood samples (1 mL) were withdrawn from the LV into plain tubes for serum samples and EDTA-coated tubes for plasma samples. Blood samples were separated by centrifugation at 2926 × g for 10 minutes at 4°C. 17β-E₂ levels were measured with the use of a radioimmunoassay kit ( Estradiol 6 Coat-a-Count, Inter Medico), and ET-1 levels were measured with the use of a sandwich ELISA kit obtained from The Next Generation Endothelin Elisa (1-21) American Research Products Inc.
Statistical Analysis
All values are expressed as mean±SEM, and n indicates the number of animals studied. Unpaired 2-tailed Student t tests were performed to compare the mean values between the groups, and when appropriate, 1-way ANOVA was used, which was followed by Student-Newman-Keuls post hoc subgroup testing if significant. Differences in mortality rates were compared by using the z test. Statistical significance was accepted at \( P < 0.05 \).

Results
Animals were shown to be adequately replaced with estrogen: serum levels of 17\( \beta \)-E\(_2\) in the oophorectomized 17\( \beta \)-E\(_2\)–treated group, measured at 10 to 11 weeks after infarct, were similar to those in animals with intact ovaries, being \( \pm 2.5\)-fold higher than the placebo-treated oophorectomized group (\( P < 0.001 \)) (Figure 1). Animals receiving 17\( \beta \)-E\(_2\) had significantly lower body weights than did those treated with placebo after 10 to 11 weeks after MI, despite there being no difference in weight among any of the groups at the start of the study (Table 1). Although mean arterial pressure was not significantly different between the treatment groups, placebo-treated rats consistently displayed the highest pressures (Table 1).

Acute Effects of MI
In the LCA-ligated rats, estrogen replacement was associated with a trend toward increased mortality, although this failed to reach statistical significance (\( P = 0.25 \)) (Table 2). No deaths occurred in the sham-operated groups. Infarct size was significantly increased in the 17\( \beta \)-E\(_2\)–treated group compared with the placebo group (42±2% versus 27±3%, respectively; \( P = 0.01 \)) (Figures 2 and 3). The ratio of scar length to body weight was calculated to exclude the potential influence of differences in body weight on infarct size, and statistical significance was still achieved when values from the 17\( \beta \)-E\(_2\)–treated and placebo-treated groups were compared (\( P = 0.009 \)) (Table 2).

Chronic Effects of MI
MI resulted in a decrease in the ratio of cavity area to septal thickness in the 17\( \beta \)-E\(_2\)–treated group compared with the sham-operated group (\( P < 0.01 \)) (Table 2). Conversely, LVs from placebo-treated animals tended to display an increased area-to-thickness ratio after MI (\( P = 0.06 \)) (Table 2). Different mechanisms were responsible for the observed changes in ratio after MI: a doubling of septal thickness (\( P < 0.001 \)) accounted for the decreased ratio in the 17\( \beta \)-E\(_2\) group, whereas a 2-fold increase in cavity area (\( P = 0.005 \)) gave rise to an increased ratio in the placebo group (Table 2).

After MI, peak wall tension was significantly lower in the estrogen-treated group compared with the placebo-treated group (419±41 versus 946±300 mm Hg/mm, respectively; \( P = 0.039 \)) (Figure 4). No significant differences in \( +dP/dt \) or \( -dP/dt \) were observed between the groups (Table 3).

| TABLE 2. Mortality Data and Morphological Measurements at 10–11 Weeks After MI |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | 17\( \beta \)-E\(_2\) | Intact | Placebo |
|                                | Sham | MI | Sham | MI | Sham | MI |
| Acute mortality (\( \leq 24 \) h), % | 0 | 48 | 0 | 48 | 0 | 27 |
| Scar length/body weight, mm/g\( \times 10^3 \) | NA | 37.5±4.0* | NA | 30.8±5.3 | NA | 20.2±3.1 |
| Cavity area, mm\(^2\) | 26±2 | 34±2 | 26±2 | 31±6 | 18±1 | 40±5† |
| Septal thickness, mm | 1.3±0.0 | 2.5±0.1† | 1.7±0.3 | 2.3±0.2 | 2.2±0.1 | 2.1±0.3 |
| Area/thickness ratio, mm\(^2\)/mm | 19±1 | 13±1† | 16±3 | 14±4 | 8±1 | 22±5 |

Values are mean±SEM. NA indicates data not applicable.
*\( P < 0.01 \) vs respective placebo group; †\( P < 0.01 \) vs respective sham group.

Figure 2. Percent relative size of infarct according to treatment group (n=6 per group). **\( P < 0.01 \) vs placebo.

Figure 3. Representative mid-LV sections 10 to 11 weeks after MI. A, 17\( \beta \)-E\(_2\)–treated rat. B, Gonadally intact rat. C, Placebo-treated rat. D, Sham-operated rat. Trichrome stain was used.
Plasma ET-1 and Cardiopulmonary Prepro-ET-1 mRNA Expression

Two weeks after hormone replacement therapy (HRT), plasma ET-1 levels did not differ between the 17β-E2 and placebo groups (P=0.39) (Figure 5). Ten to 11 weeks after MI, the plasma ET-1 concentration of the MI rats was 5-fold (P=0.046) higher than that in the sham-operated rats for the 17β-E2 group (Figure 5). The rise in ET-1 levels in placebo-treated rats after MI did not reach statistical significance compared with levels in the sham-operated group (P=0.24) (Figure 5).

Prepro-ET-1 mRNA levels in the LV were similar between the 17β-E2 and placebo groups in both sham-operated and MI rats (Table 4). The increase in prepro-ET-1 expression after MI was of borderline significance in the 17β-E2 (P=0.07) and placebo (P=0.06) groups (Table 4) (Figure 6). In the lung, prepro-ET-1 mRNA levels did not differ between hormone-treatment groups or infarct/sham-operated rats (Table 4) (Figure 6).

Cardiopulmonary ET_A and ET_B Receptor Expression

ET_A mRNA expression did not significantly change after MI in 17β-E2–treated and placebo-treated animals, in either the LV (Figure 7a) or lung (Figure 7b), nor were there any differences between the 17β-E2 and placebo groups for sham-operated or MI animals. Cardiac ET_B mRNA expression was increased 3-fold in the LV of placebo-treated rats with MI compared with sham-operated rats (P=0.004, Figure 8a). The MI-induced increase in ET_B mRNA expression was absent in 17β-E2–treated animals (Figure 8a), and there was a decrease in pulmonary ET_B mRNA expression after MI in 17β-E2–treated animals compared with sham-operated animals, which was not prevented by 17β-E2 replacement (P=0.023, Figure 8b).

The increase in ET_B mRNA after MI in placebo-treated rats correlated with a marked increase in myocardial ET_B receptor expression as determined by immunohistochemistry (Figure 9) and immunofluorescence (Figure 10). Estrogen replacement prevented this increase, in agreement with the mRNA data. Therefore, the upregulation of ET_B after MI can be explained by changes in the expression of this receptor on the cardiac myocytes, inasmuch as there was no difference in the abundant expression of ET_B receptors in the scar tissue between estrogen- and placebo-replaced animals. Of interest, even the myocytes interspersed in the scar tissue showed evidence of this differential level of expression. There was no obvious difference between the 2 experimental groups in the expression of ET_B on the vascular smooth muscle cells or the pericardium. Negative controls (without antibody) showed a signal that was no different from background (data not shown).

Discussion

The results of the present study suggest that physiological levels of estrogen are associated with significant cardiac effects after MI in female rats. Acutely, estrogen was associated with an increase in the size of infarct and a trend toward increased mortality. Chronically, estrogen inhibited LV dilatation and normalized peak wall tension in MI survivors. These findings are consistent with epidemiological

<table>
<thead>
<tr>
<th>TABLE 3. Hemodynamic Measurements at 10–11 Weeks After MI</th>
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<tr>
<td>17β-E2</td>
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<tr>
<td>--------</td>
</tr>
<tr>
<td>HR, bpm</td>
</tr>
<tr>
<td>Peak LVSP, mm Hg</td>
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<tr>
<td>LV + dP/dt, mm Hg/s</td>
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<td>LV – dP/dt, mm Hg/s</td>
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</tbody>
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Values are mean±SEM. HR indicates heart rate; LVSP, LV systolic pressure; LV + dP/dt, positive first derivative of LV pressure; and LV – dP/dt, negative first derivative of LV pressure.
and clinical data regarding sex differences in outcome after MI, ie, a potential short-term detriment, particularly in women aged 30 to 49 years who are premenopausal and perimenopausal,2,3,5 in contrast to a long-term benefit in survivors.4,5

Acute Effects
The process of scar formation takes \( \sim 3 \) weeks in the rat and involves the deposition of extracellular matrix, which serves to limit infarct expansion.11 Functional estrogen receptors are present on cardiac fibroblasts,15 and estrogen has been shown to inhibit the formation of collagen in noncardiac cells.16 Furthermore, estrogen can inhibit the growth of cardiac fibroblasts.17 The suppression of fibroblast growth and collagen synthesis may be in part responsible for the increased scar size found in the 17\( \beta \)-E\(_2\)-treated rats. In addition to its direct effect on collagen synthesis, estrogen may inhibit the response to other factors that are normally upregulated after MI and that, by themselves, increase extracellular matrix formation, eg, ET-1.18 In fact, the early administration of an ET receptor antagonist in the same rat MI model has led to infarct expansion in a manner similar to our results,13 whereas delayed ET blockade has been shown to be beneficial.19 Presently, we do not have an explanation for the observation that acute mortality tended to be higher in those animals receiving estrogen. Ventricular arrhythmias are the primary acute cause of mortality after MI; however, estrogen is considered to have antiarrhythmic properties.20 A possible hypothesis to explain our findings is that estrogen attenuates or downregulates a number of stress responses that are important to overcome the acute insult or limit its consequences. As mentioned, ET would be but one example.

Chronic Effects
LV cavity volume has been shown to be a major predictor of mortality in congestive heart failure (CHF). There are numerous potential mechanisms whereby estrogen may prevent the adverse LV remodeling after MI. A reduction in preload and afterload can attenuate cardiac remodeling as a result of a decrease in myocardial wall stress. Estrogen acts as a potent vasodilator, through nongenomic antagonism of L-type calcium channels21 and through upregulation of vasodilator pathways, such as NO.22 In the present study, mean arterial pressure was lowest in rats with intact ovaries and those treated with 17\( \beta \)-E\(_2\), suggesting (among other mechanisms) a vasodilatory role of estrogen that could presumably have resulted in a decreased preload and afterload. In addition, estrogen can inhibit ACE activity,23 decrease the response to adrenergic stimuli,24 and increase parasympathetic tone,25 all of which might be expected to have a long-term beneficial effect.

Estrogens have the potential to influence myocardial gene expression of cardiac growth factors and cytokines that are upregulated after MI and subsequent CHF.15 ET-1 is an example of a hypertrophic factor implicated in the pathogenesis of CHF. If ET is a major contributor to the pathophysiology of MI and CHF, one may hypothesize that the increased infarct size and reduced ventricular remodeling found in estrogen-treated animals is due, in part, to the interaction between ET and estrogen. The most striking finding in the present study was the effect of estrogen on the expression of ET\(_B\) receptor mRNA in the LV. Whereas MI

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**Table 4. Tissue Prepro-ET-1 mRNA at 10–11 Weeks After MI**

<table>
<thead>
<tr>
<th></th>
<th>17( \beta )-E(_2) Placebo</th>
<th></th>
<th>17( \beta )-E(_2) Placebo</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sham MI</td>
<td>Sham MI</td>
<td>Sham MI</td>
</tr>
<tr>
<td>LV</td>
<td>0.50±0.03</td>
<td>0.59±0.03</td>
<td>0.50±0.02</td>
</tr>
<tr>
<td>Lung</td>
<td>0.79±0.08</td>
<td>0.73±0.04</td>
<td>0.73±0.06</td>
</tr>
</tbody>
</table>

Values are mean±SEM and are ratios of levels of prepro-ET-1 to \( \beta \)-actin mRNA.

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**Figure 6.** Representative ribonuclease protection assay of prepro-ET-1 and \( \beta \)-actin mRNA expression in LV and lung.13 1 indicates placebo sham; 2, placebo MI; 3, 17\( \beta \)-E\(_2\) sham; and 4, 17\( \beta \)-E\(_2\) MI.

**Figure 7.** Competitive reverse transcription–PCR assessment of effects of estrogen treatment on ET\(_B\) mRNA expression in LV (A) and lung (B). Open bars represent sham-operated group (n=5); shaded bars represent MI group (n=6 or 7). Representative gels were from 17\( \beta \)-E\(_2\) (left) and placebo (right) MI samples; 5 serial dilutions of ET\(_A\) mutant were used to compete 2 \( \mu \)g total RNA. PCR products were digested with EcoRV for 3 hours to distinguish wild type (232 bp) from mutant (131 and 101 bp).14
induced a 3-fold increase in ET\(_B\) expression in placebo-treated rats, estrogen suppressed this increase by preventing
selectively the myocardial upregulation of ET\(_B\), with no effect
on other cell types. The mechanism whereby estrogen inhibits
the upregulation of ET\(_B\) expression is unclear from the present
data. It could be through direct transcriptional regulation of
the ET\(_B\) gene itself or through altered expression of another
gene(s), which, in turn, regulates the ET\(_B\) receptor. For
instance, angiotensin II has been reported to increase ET\(_B\)
mRNA expression in cultured myocytes.\(^{26}\) This effect is
mediated by angiotensin type 1 receptors, which may them-
selves be modulated by estrogen: recent studies in rat vascul-
lar smooth muscle cells reveal that treatment with 17\(\beta\)-E\(_2\)
markedly downregulates angiotensin type 1 receptor expres-
sion.\(^{27}\) However, the possible role played by the renin-
angiotensin system in our findings is beyond the scope of the
present study.

ET\(_B\) receptors in the LV have been described on cardiac
myocytes, fibroblasts, and vascular smooth muscle cells of
coronary arteries. We also found them on the pericardium.
Only myocardial ET\(_B\) receptors were increased after MI in the
absence of estrogen, whereas there was no change in the other

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**Figure 8.** Competitive reverse transcription–PCR assessment of
effects of estrogen treatment on ET\(_B\) mRNA expression in LV (A)
and lung (B). Open bars represent sham-operated group (n=5); shaded bars represent MI group (n=6 to 8), **P<0.01 vs group
indicated. Representative gels were from 17\(\beta\)-E\(_2\) (left) and pla-
cese (right) MI samples; 5 serial dilutions of ET\(_B\) mutant were
used to compete 2 \(\mu\)g total RNA. Because templates differed by
105 bp, wild-type fragment (553 bp) was able to be distin-
guished from mutant (448 bp) immediately after PCR.\(^{14}\)

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**Figure 9.** Localization of ET\(_B\) by immunohistochemistry in rat
hearts after MI. a and d, Picrosirius red staining of transitional
zone between scar tissue (stained red) and noninfarcted myo-
cardium (stained yellow) is shown in placebo- and estrogen-
replaced rats, respectively (original magnification \(\times 100\)). b and
e, Immunostaining of adjacent sections of same hearts with ET\(_B\)
antibody is shown. Note presence of more intense immuno-
staining (brown) for ET\(_B\) in noninfarcted myocardium of placebo-
replaced rat (b) compared with myocardium of estrogen-
replaced rat (e). c and f, This can be seen more clearly under
higher magnification in placebo-replaced (c) and estrogen-
replaced (f) rats (original magnification \(\times 200\)).

**Figure 10.** Immunolocalization of ET\(_B\) by confocal microscopy. a
and b, Confocal photomicrographs of scar tissue showing abun-
dant ET\(_B\) expression (bright green) in cells of this region. Arrow
indicates ET\(_B\) staining on endothelial cells of blood vessel. c and
d, Confocal photomicrographs of ET\(_B\) localization on cardiac
myocytes. Note strong staining for ET\(_B\) (bright green, punctate
pattern) on myocytes from placebo-treated heart (c) compared
with estrogen-replaced heart (d). Bar=250 \(\mu\)m.
cell types. An increased expression of ET<sub>B</sub> receptors on myocytes could favor hypertrophy, dilatation, and increased wall stress. Given that placebo-treated rats displayed dilatation of the LV and an increase in peak wall tension, concomitant with increased ET<sub>B</sub> mRNA expression, one may speculate that the receptor upregulation on the myocytes could have played a pathophysiological role in this process.

We found no effect of hormone treatment on plasma ET-1 levels at either 2 weeks or 12 to 13 weeks after pellet insertion in noninfarcted rats. Although some studies show that HRT reduces levels in postmenopausal women, this has not been a consistent finding. Indeed, a recent study reported that HRT did not affect plasma ET-1 levels in healthy nonsmoking postmenopausal women but reduced them in postmenopausal smokers displaying high initial levels before treatment. These data suggest that estrogens modulate ET-1 levels when the ET system is activated by certain stimuli but not under basal conditions.

The results from the present study may have clinical relevance for the treatment of cardiovascular disease in women. Because estrogen replacement was associated with short-term detriment after MI but long-term beneficial effects, our results could have implications for the management of acute MI in premenopausal women, in women taking HRT, and in the long-term management of women with LV dysfunction. Our results are consistent with, and may provide some of the mechanisms to account for, the observed sex differences in outcomes after MI, which recently have been established to be more pronounced in younger women and are therefore, likely to be influenced by hormonal status.

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