Estrogen Receptor β Protects the Murine Heart Against Left Ventricular Hypertrophy

Fawzi A. Babiker, Daniel Lips, Rainer Meyer, Els Delvaux, Pieter Zandberg, Ben Janssen, Guillaume van Eys, Christian Grohé, Pieter A. Doevendans

Background—Left ventricular hypertrophy (LVH) displays significant gender-based differences. 17β-estradiol (E2) plays an important role in this process because it can attenuate pressure overload hypertrophy via 2 distinct estrogen receptors (ERs): ERα and ERβ. However, which ER is critically involved in the modulation of LVH is poorly understood. We therefore used ERα-deficient (ERα−/−) and ERβ-deficient (ERβ−/−) mice to analyze the respective ER-mediated effects.

Methods and Results—Respective ER-deficient female mice were ovariectomized and were given E2 or placebo subcutaneously using 60-day release pellets. After 2 weeks, they underwent transverse aortic constriction (TAC) or sham operation. In ERα−/− animals, TAC led to a significant increase in ventricular mass compared with sham operation. E2 treatment reduced TAC induced cardiac hypertrophy significantly in wild-type (WT) and ERα−/− mice but not in ERβ−/− mice. Biochemical analysis showed that E2 blocked the increased phosphorylation of p38–mitogen-activated protein kinase observed in TAC-treated ERα−/− mice. Moreover, E2 led to an increase of ventricular atrial natriuretic factor expression in WT and ERα−/− mice.

Conclusions—These findings demonstrate that E2, through ERβ-mediated mechanisms, protects the murine heart against LVH. (Arterioscler Thromb Vasc Biol. 2006;26:1524-1530.)

Key Words: hypertrophy ■ hormones ■ myocardium ■ gender

The increase of left ventricular mass represents a structural mechanism of compensation of the heart in response to pressure overload. The resulting left ventricular hypertrophy (LVH) is an important, independent negative predictor of cardiac morbidity and mortality. LVH displays significant gender-based differences. Premenopausal women have a lower prevalence of LVH than men. The Coronary Artery Risk Development In young Adults (CARDIA) study demonstrated a higher prevalence of LVH in men, even after correction for a large number of risk factors. It further demonstrated that the difference in left ventricular size begins early in life (ie, before menopause), suggesting that intrinsic factors are involved in the induction of LVH. Sex hormones such as estrogen have been attributed to play an important role in the pathogenesis of cardiovascular disease. The recent clinical trials with respect to the therapeutic role of 17β-estradiol (E2) vascular disease are controversial. However, the potential of E2 as a therapeutic option in the modulation of cardiac disease remains poorly understood. It has been demonstrated that estrogens are able to attenuate hypertrophic responses. E2 appears to act as a cardioprotective steroid hormone. However, the underlying mechanisms of E2 protection of the myocardium are not fully understood. Myocytes and fibroblasts contain functional estrogen receptors (ERs) ERα and ERβ. Via these receptors, E2 modulates the activity of the mitogen-activated protein kinase (MAPK) pathways in cardiac myocytes. The MAPK signaling pathways consist of a sequence of successively acting kinases that ultimately result in the dual phosphorylation and activation of effector kinases such as p38-MAPKs, c-Jun N-terminal kinases (JNKs), and extracellular signal-regulated kinases (ERKs), which subsequently phosphorylate a large array of targets, leading to altered gene expression patterns. These signaling cascades play an important role in the initiation of cardiac hypertrophy and in the development of heart failure. E2 can inhibit p38-MAPK phosphorylation and thus p38-MAPK activation. Furthermore, it is known that E2 can increase the expression of the atrial natriuretic factor (ANF), which recently has been shown to possess antihypertrophic effects. Significant increases in ANF mRNA are detected in the mouse ventricle that is challenged by aortic banding. However, little is known about the respective role of the...
distinct ERs. We recently reported the effects of E2 on the development of pressure-overload hypertrophy and the activation of signaling pathways of MAPKs. Furthermore, new studies suggest that ERβ plays an important role in cardiac disease. Here, we further define the role of ERs in this process. For this goal, we used ERα-deficient (ERα−/−) and ERβ-deficient (ERβ−/−) mice. We found that cardioprotective effects of E2 on LVH are mediated by ERβ and not ERα. These effects are paralleled by an increase in the expression of ANF and a decrease in the phosphorylation of p38.

Materials and Methods

Animals
ERα+/ transgenic mice were generated using C57BL/6 as background as described previously. These mice, which have been extensively studied, do not express ERα protein in any tissue. ERβ+/ mice were generated and provided by Organon (Oss, the Netherlands). For details, see the online supplement, available at http://atvb.ahajournals.org. Mice showing germline transmission were again crossed with C57BL/6 mice (F2+/F3 generation). Subsequently, mice from the same litters were used for breeding. Wild-type (WT) littermates from the respective genotypes were used in the study. Only female mice of ~10 weeks age were incorporated into this study.

Experimental Procedures
All animals (300 WT and knockout mice) were housed under standard conditions. Animals were anesthetized with ketamine (100 mg/kg body weight [BW] IP) and xylazine (10 mg/kg BW IP) for ovariectomy, pellet placement, and transverse aortic constriction (TAC). The study was approved by the animal ethics committee of the University of Maastricht.

Estrogen Replacement
Two weeks after ovariectomy, a 60-day-release pellet containing 0.18 mg E2 or placebo was implanted subcutaneously. All pellets were purchased from Innovative Research of America. E2 serum levels were measured with a radioimmunoassay (DPC Biermann) in a subset of animals.

Surgical Procedures and Hemodynamics
Ovariectomy was performed by a standard bilateral abdominal approach. The uterus was left remaining to study the responsiveness to hormone replacement therapy. Afterward, placebo or E2-containing pellets were implanted in the upper neck subcutaneously. Two weeks after the pharmacological intervention, TAC was performed, as described previously. Sham-operated animals underwent an identical operation without placement of the constricting suture. Assessment of left ventricular function was performed as described previously. Conductance and pressure input was digitized with a Conduct-PC data acquisition system (CDLeycom BV). Average values for mean arterial pressure, heart rate, systolic and diastolic LV pressure, and left ventricular end-diastolic pressure were determined. The mortality in all treatment groups during the surgery did not differ significantly between groups. In particular, no increased mortality was found in the estrogen treatment groups and the ERβ animal groups.

Tissue Preparation and Histology
Hearts were arrested in diastole with CdCl2 (0.1 mol/L IV). For morphometric analysis, hearts were fixed in 10% formalin and embedded in paraffin as described previously. For protein extraction, hearts were excised and washed in ice-cold PBS. All external fluid was completely removed before the organs were weighed and frozen. Transverse sections of the heart were stained with hematoxylin and eosin, sirius red, or modified Azan. The analysis of the collagen content was performed with a computerized morphometry system as described previously.

Immunoblot Analysis
Total heart lysates (40 μg per lane) were analyzed by standard immunoblotting procedures as described previously. For details, see the online supplement.

Real-Time Polymerase Chain Reaction Analysis
Details of the real-time RT-PCR have been described previously. The primer sequences used for real time PCR are: ANF 5’ primer (5’-CCT GTG TAC AGT GGC GTG TC), ANF 3’ primer (5’-TCC AGG TGG TCT AGC A), alpha-actin 5’ primer (5’-CAA ATG CTG GAC CAA ACA CAA), cyclophilin 3’ primer (5’-TTC ACC TTC CCA AAG ACC ACA T). The CT measurement is defined at the fractional cycle number at which the amount of amplified target reaches a fixed threshold above background Sybr Green fluorescence. The amount of target in the cDNA sample relative to cyclophiline was calculated.

Statistical Analysis
Data are shown as mean±SEM. Means were compared by ANOVA, followed by Bonferroni test for multiple comparisons. Differences were considered significant at P<0.05.

Results
In our study, we divided the cardiac analysis of the animals in a total of 16 different groups, as shown in the Table. The different groups underwent either sham or TAC surgical procedures and were ovariectomized after E2 substitution. A complete phenotypic analysis of both cardiac and endocrine parameters was performed to study the receptor-mediated effects in all animal groups studied (Table). E2 replacement led to a reconstitution of physiological E2 levels (122 pg/mL in E2-treated versus <5 pg/mL in placebo-treated). Uterus weight (UW) was measured to demonstrate the effectiveness of ovariectomy and E2 substitution in all animals. In all groups (8 conditions with TAC or sham and placebo or E2 treatment for ERα−/− as well as ERβ−/−), the UW/BW and UW/tibia length (TL) ratios showed a significant difference between placebo and E2-treated mice (Table). In E2-treated WT and ERβ−/− mice, the UW/BW ratios are significantly higher than that of E2-treated ERα−/− mice (Table). Together, we were able to demonstrate that ovariectomy leads to uterus atrophy on the basis of E2 withdrawal, and E2 replacement restored UW. There were no significant differences in BW between the groups and no significant changes in lung weight (Table).

In all animal groups, TAC led to a significant increase in ventricular mass 4 weeks after the intervention. E2 treatment led to a significant reduction of the increase of ventricular weight (VW) and the VW/TL ratio in WT and ERα−/− mice (Figure 1). No differences were observed between sham-operated mice (Figure 1; data not shown). Also in ERβ−/− mice and their WT littermates, TAC led to significant increase in ventricular mass 4 weeks after the intervention. In WT mice, TAC the degree of ventricular hypertrophy were significantly lower in E2-treated compared with placebo-treated mice. Interestingly, E2 treatment in ERβ−/− mice resulted in a higher level of hypertrophy compared with WT mice. Similar results were found when we used VW/BW (please see the online supplement; data not shown). No
Effects of TAC and E2 Treatment on BW and Organ Weight

<table>
<thead>
<tr>
<th></th>
<th>WT (n= 7)</th>
<th>E2 (n= 10)</th>
<th>TAC (n= 10)</th>
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<td>VW, mg</td>
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<tr>
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<td>6.46 ±0.20</td>
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<tr>
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<td>ERα−/− (n= 7)</td>
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<td>VW, mg</td>
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<tr>
<td>VW/BW, mg/g</td>
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<tr>
<td>VW, mg</td>
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BW indicates body weight; VW, ventricular weight; TL, tibial length; UW, uterus weight. All values are Mean ± SEM.

significant differences were observed among the sham-operated mice (Figure 1). Weight analyses are in line with morphometric analyses (please see the online supplement).

The most surprising finding was the lack of inhibition of hypertrophy in the ERβ−/− mice. Therefore, we performed invasive hemodynamic measurements to check whether the blunted response had an effect on left ventricular function. Hemodynamic analysis showed that developed pressure, as an indication of the quantity of afterload, was significantly increased in TAC ERα−/− and ERβ−/− instrumented mice compared with sham mice (please see the online supplement). E2 treatment had no influence on the degree of pressure overload compared with placebo-treated mice (please see the online supplement). There was no significant difference.
between ERα−/− and ERβ−/− mice. Cardiac performance remained at normal WT level because no significant differences in cardiac output and heart rate were detected between groups (please see the online supplement). Even in the ERβ−/− mice, no deterioration of cardiac function was observed after 4 weeks of pressure overload. Hypertrophic growth is therefore not necessary to maintain cardiac performance in the situation of pressure overload. This is not in accordance with the general consensus that hypertrophy is an obligatory compensating mechanism to withstand augmented hemodynamic stress. In this study, ventricular contraction and relaxation did not alter between genotypes nor substitution therapies after TAC. Maximal derivative of left ventricular pressure (dP/dtmax) increased and minimal derivative (dP/dtmin) decreased, although no significant differences were found between groups (Figure 2). The pressure volume loops showed the absence of significant differences in cardiac performance between placebo- and E2-treated ERβ−/− (Figure 2), except for the TAC-induced systolic pressure rises. Data in ERα−/− mice are comparable (data not shown). E2 had no influence on the degree of pressure overload as determined by the pressure gradient or the prestenotic pressure (data not shown).

To analyze the mechanisms involved in the observed antihypertrophic effect of E2, we investigated critical target genes involved in development and progression of cardiac hypertrophy. In previous reports, these genes have been shown to be regulated by E2.4 Immunoblot analysis revealed that E2 blocked the increased phosphorylation of p38-MAPK in ovariectomized WT and ERα−/− mice with pressure overload hypertrophy, whereas it exerted no effect in sham-operated animals and ERβ−/− mice (Figure 3). No differences could be observed between the study groups with regard to the phosphorylation level of ERK1/2 and JNK (data not shown).

Furthermore, TAC led to a significant increase in ANF expression in the hypertrophied ventricles of WT and ERβ−/− mice 4 weeks after intervention compared with placebo-treated and ERβ−/− mice (Figure 3). No such effect was found (Figure 3). No significant difference was seen between placebo-treated animals in both groups. Also, E2-treated WT mice showed a nonsignificant difference between E2-treated WT of ERα−/− and ERβ−/− animals (Figure 3).

**Discussion**

ER-mediated effects in cardiovascular disease require a better understanding because of the controversial findings of previous clinical studies of hormone replacement therapy.25 These prospective studies focused on primary and secondary prevention of ischemic heart disease. Despite these recent observations, little is known about the role of ERs in cardiac disease such as cardiac hypertrophy. A better understanding of the function of specific ERs in different tissues is important in the development and selection of new agents that could be used for treatment. Currently, the biological roles of these 2 different ER subtypes are not clear. It may be related to the selective actions of E2 in various target tissues. Also, it is known that different E2 compounds have different

**Figure 2.** Cardiac function measured in ERβ−/− and WT mice. Representative analysis of cardiac output (CO; A), dP/dtmax (B), and dP/dtmin (C). D shows left ventricular in vivo pressure–volume loops in sham placebo-treated WT mice (ERβ−/− littermates; black loops), TAC placebo-treated ERβ−/− mice (blue loops), and TAC E2-treated ERβ−/− (red loops). RVU indicates relative volume units. Pressure gradients of all groups are shown. All values are mean±SEM; n=7 for sham and 10 for TAC per group.
relative binding affinities for ERα versus ERβ. For instance, recent studies suggest that ERβ may inhibit the stimulatory effects of ERα on cellular proliferation. In the case of E2 signaling, cellular selectivity for one or the other ER appears to be regulated by the cellular expression pattern of the ERs and interacting coactivator and corepressor proteins. The ERα appears to be more involved in regulation of uterine growth than the ERβ, as can be deduced from both the uterine wet weight and the uterine dry weight in ERα/− mice (Table). This is in line with previous studies regarding the importance of ERα for the uterine response. On the other hand, ERβ, as we proved in this study, mediates the attenuation of pressure overload hypertrophy by E2. Because of the overall number required to study all treatment groups to reach statistical significance, we focused on the well-established time point of 4 weeks after TAC.

Whereas in ERα/− mice, VW is significantly reduced (comparable to WT) after addition of E2, no such effect is seen in ERβ/−. Moreover, in ERβ/− mice, there was a nonsignificant tendency toward decreased hypertrophy when E2 was present and a tendency toward increased hypertrophy when E2 was absent. Together, our study supports the hypothesis that E2 has direct, modulating effects on cardiac myocytes and the heart. Similar results were also obtained by Skavdahl et al in a model of hypertrophy that evaluated gender-based differences and added the important observation that gender determines the hypertrophic phenotype. Furthermore, Pelzer et al demonstrated that in ERβ/− animals of a different genetic background than the animals used in our study, an increase of mortality was shown. The findings of Pelzer et al underline the importance of ERβ for the cardiovascular system, in particular for cardiac dimensions and function. Although ERβ appears to be of major importance in the ER-dependent responses studied in this investigation, ERβ also plays a role in other physiological contexts such as the development of cardiac arrhythmias after myocardial infarction. There, ERβ was shown to play an important role in ventricular repolarization after myocardial infarction and the regulation of the potassium channel expression. Furthermore, it was demonstrated, using the same animal model, that ERβ is necessary for normal morphology in several regions of the

![Figure 3](image-url)
central nervous system. Studies on these animals also indicate that ERβ has an antiproliferative effect in the immature uterus and in the prostate, at least partially by balancing the proliferative activity of ERα. To further elucidate the mechanisms involved in the antihypertrophic effects of E2, it will be necessary to identify the additional signaling molecules involved in these protective effects, their time course of activation, and the cross-talk between them.

In a previous study, we showed that no differences occur in the expression levels of ERK1/2, JNK, angiotensin II type 1 receptor, or angiotensin-converting enzyme. To further elucidate possible mechanisms involved, we studied the activation of MAPK and ANF. These have been shown to play important roles in the development and progression of cardiac hypertrophy. It has been reported that the activation of p38-MAPK is important for the hypertrophic response and maintains the hypertrophic response over a longer period of time. E2 can inhibit p38-MAPK phosphorylation and thus p38-MAPK activation. Our results are in line with van Eickels et al., who demonstrated that inhibition of p38-MAPK phosphorylation by E2 treatment may represent one of the mechanisms by which E2 exerts its antihypertrophic effect in the TAC model of pressure overload.

Antihypertrophic properties of ANF were shown in several studies. In line with these results, E2 led to a significant increase in ANF expression in the ventricles of WT and ERα−/− animals compared with placebo-treated WT and E2-treated ERα−/− mice with pressure overload. ANF expression was noticed early after hypertrophic stimulation; it occurs after 6 to 12 hours. These findings confirm that ANF is part of another pathway by which E2 exerts its antihypertrophic effects. Together, we provide new evidence for the role of cardiac ERs in the development of LVH.

However, the role of E2 in the development of LVH is poorly understood. Many observational studies suggest that estrogen replacement therapy has cardioprotective effects in postmenopausal women. However, recent clinical trials have failed to show a cardiovascular benefit of estrogen replacement therapy in women with established coronary artery disease. Our results show that the antihypertrophic effects of E2 are receptor specific. This may stimulate research for ligands that only bind ERβ. Such a development may circumvent the negative effects reported in the Heart and Estrogen Replacement Study (HERS) and Women Health Initiative Study (WHI) studies and provide for a precisely targeted antihypertrophic approach.

Conclusion
Our results showed that ERβ plays a role in the control of LVH. Protective effects of E2 in murine heart via ERβ appear to increase expression of ANF and decreased p38 phosphorylation. The fact that cardiac action of E2 relies largely on ERβ provides opportunities to develop more specific interventional strategies to treat hypertrophy, avoiding side effects.

Acknowledgments
We thank Organon, Oss, the Netherlands for providing the animals.

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Disclosures
None.

References
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Material and Methods

Animals

The mouse ERβ gene was isolated from an 129 Sv genomic BAC library (Genome Systems) by screening with a full length human ERβ cDNA probe. Two BAC clones were obtained and restriction enzyme mapping was performed using a panel of restriction enzymes and degenerated probes deduced from exon 1-4. Two fragments were identified: a 2.7 kb HindIII-EcoRV fragment located 5' of exon 1 and a 1.6 kb HindIII fragment located 3' of exon 2. Both fragments were cloned into the pKO gene targeting vector (Lexicon Genetics, Woodlands, USA) containing the pgk-neo gene for positive selection and the CMV-Tk gene for negative selection. ES cells were transfected with NotI linearized targeting vector using a BioRad Gene Pulser(230V). Targeted ES cells were identified by nested PCR analysis. PCR conditions used were 100 ng each of primer 5'-GGAGTGGCAGACAAGGGCA-3' and primer 5'-GATTGCAGCGCAGATCGCC-3', 10 ng genomic DNA, DNA Taq polymerase(1U, Gibco BRL), a dATP, dCTP, dGTP, dTTP mixture (10 mM), Taq buffer (Gibco BRL) for 20 cycles. Nested PCR was performed using the nested primers 5'-ACCCAACACCTGCTCGGACC-3 and 5'-GGGCTCTATGCTTCTGAGG-3' for 24 cycles. PCR products were analyzed on an 1% agarose gel and visualized with ethidium bromide. Positive clones obtained were analyzed by Southern blot analysis. Targeted clones were expanded and injected into blastocysts from C57Bl/6 mothers (Genome Systems), and where returned to pseudopregnant C57Bl/6 hosts to complete their development. Germ line transmission of the mutant allele was tested by PCR analysis of genomic tail DNA.
Immunoblot Analysis

Equal loading was checked by stripping and reprobing the membrane with troponin C. The following primary antibodies were used: p38-mitogen-activated protein kinase (p38-MAPK), ERK1/2, JNK, phospho-JNK (Thr183/Tyr185) and troponin C (Santa Cruz Biotechnology Inc) and phospho-ERK1/2 (Thr202/Tyr204) and phospho-p-38 MAPK (Thr180/Tyr182) (New England Biolabs). Detection was performed with the enhanced chemiluminescence technique after incubation with a suitable secondary antibody coupled to horseradish peroxidase (ECL; Amersham Pharmacia Biotech). A computerized image acquisition system (Alpha Innotech Corp) was used for densitometric analysis.

Figure 1. Transverse sections of hearts stained with hematoxylin-eosin from WT and ERα−/− animals with TAC that where treated with either E2 or placebo 4 weeks after intervention (A). Transverse sections of hearts stained with hematoxylin-eosin from WT and ERβ−/− animals with TAC that where treated with either E2 or placebo 4 weeks after intervention (B). Left ventricular mass analysis for WT and ERα−/− (C). Left ventricular mass analysis for WT and ERβ−/− (D). Ventricular (LV) external circumference analysis for WT and ERα−/− (E). Ventricular external circumference analysis for WT and ERβ−/− (F): All values are mean ± SEM, n = 7 for sham and 10 for TAC per group. *Indicates P<0.05 for placebo vs. E2 treatments.
Figure II. Cardiac function measured in ERα⁻/⁻ and ERβ⁻/⁻ and wild type littermates. Figure A and B show measurements of developed pressure in sham versus TAC treated animals with and without E2 tretament. Figure C and D show heart rate monitoring in these animals. All values are mean ± SEM, n = 7 for sham and 10 for TAC per group. †indicates P < 0.05 TAC vs. sham.
Cardiac histological analysis of ERα⁻/⁻ and ERβ⁻/⁻ mice

Left ventricular mass in TAC ERα⁻/⁻ and ERβKO models

Left ventricular circumference in TAC ERα⁻/⁻ and ERβKO mice
Figure II

Developed pressure in ERα−/− and ERβ−/− mice

A

ERα−/−

B

ERβ−/−

C

D

Heart Rate in ERα−/− and ERβ−/− mice