Upregulation of Myocardial Estrogen Receptors in Human Aortic Stenosis

Johannes Nordmeyer, AS*; Sarah Eder, AS*; Shokoufeh Mahmoodzadeh, PhD; Peter Martus, PhD; Jens Fielitz, MD; Jan Bass, AS; Nicole Bethke, AS; Heinz R. Zurbrügg, MD; Reinhard Pregla, MD; Roland Hetzer, MD; Vera Regitz-Zagrosek, MD

Background—Estrogen receptor (ER)–mediated effects have been associated with the modulation of myocardial hypertrophy in animal models and in humans, but ER expression in the human heart and its relation to hypertrophy-mediated gene expression have not yet been analyzed. We therefore investigated sex- and disease-dependent alterations of myocardial ER expression in human aortic stenosis together with the expression of hypertrophy-related genes.

Methods and Results—ER-α and -β, calcineurin A-β, and brain natriuretic peptide (BNP) mRNA were quantified by real-time polymerase chain reaction in left ventricular biopsies from patients with aortic valve stenosis (n=14) and control hearts with normal systolic function (n=17). ER protein was quantified by immunoblotting and visualized by immunofluorescence confocal microscopy. ER-α mRNA and protein were increased 2.6-fold (P=0.003) and 1.7-fold (P=0.026), respectively, in patients with aortic valve stenosis. Left ventricular ER-β mRNA was increased 2.6-fold in patients with aortic valve stenosis (P<0.0001). ER-α and -β were found in the cytoplasm and nuclei of human hearts. A strong inverse correlation exists between ER-β and calcineurin A-β mRNA in patients with aortic valve stenosis (r=−0.83, P=0.002) but not between ER-α or -β and BNP mRNA.

Conclusions—ER-α and -β in the human heart are upregulated by myocardial pressure load. (Circulation. 2004;110:3270-3275.)

Key Words: receptors, estrogen, aortic valve, hypertrophy

Sex differences in left ventricular hypertrophy and remodeling have been observed in aging and pressure-loaded human hearts. Hearts from female patients with aortic valve stenosis are characterized by a different form of hypertrophy than male hearts. Sexual hormones and/or their myocardial receptors are first-line candidates to explain such differences.

Estrogen receptor (ER)-α and -β belong to the steroid hormone receptor superfamily, which can act as transcription factors on downstream target genes and also exert nongenomic effects. Both ERs are expressed in rat cardiac myocytes and fibroblasts as well as in the human heart. Multiple lines of evidence suggest the involvement of ERs in the development of cardiac hypertrophy in animal models. The hypertrophic growth in cardiac myocytes is regulated by intracellular calcium (Ca2+) availability. A disruption of the ER in mice leads to an increase in the expression of L-type Ca2+ channel expression in ventricular myocytes. Estrogen reduces L-type Ca2+ channel activity in vitro and protects mice from the consequences of increased intracellular Ca2+, which may lead to cardiac hypertrophy by activation of the Ser/Thr-phosphatase calcineurin.

17β-Estradiol attenuated the development of pressure-overload hypertrophy in a rat model of aortic stenosis and inhibited the phosphorylation of p38 mitogen-activated protein kinases. In the same model, 17β-estradiol increased atrial natriuretic peptide (ANP) gene expression, which was induced by pressure overload. In rat neonatal cardiac myocytes, the activation of ER induced ANP gene transcription.

Effects of estrogen on brain natriuretic peptide (BNP) gene transcription, which acts in a manner similar to ANP as a marker or modulator of hypertrophy in a number of clinical conditions, have not yet been analyzed. However, in clinical studies healthy women have higher BNP levels than healthy men, and BNP levels are increased by hormone replacement, suggesting effects of estrogen on BNP as well.

Whereas a number of studies describe the interaction of estrogen and ER with myocardial hypertrophy in animal models, only a few data are available on the human heart. A reduced degree of cardiac hypertrophy in women taking hormone replacement therapy in comparison with nonusers has been described, but molecular mechanisms underlying
normal systolic cardiac function, no history of cardiac disease, and samples of donor hearts that were rejected for logistic reasons with replacement surgery. The control group was composed of tissue study were postmenopausal, and hormone replacement therapy was women and 5 men) in a largely overlapping cohort. All women in the measured in left ventricular myocardial samples of 10 control

TABLE 1. Clinical and Hemodynamic Data in Patients Studied

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age, y</th>
<th>Systolic BP, mm Hg</th>
<th>Δp mean, mm Hg</th>
<th>LVEF, %</th>
<th>LVEDD, mm</th>
<th>FS, %</th>
<th>Diuretics, %</th>
<th>ACEI/ARB, %</th>
<th>β-Blocker, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS (female)</td>
<td>8</td>
<td>65.6±4.5</td>
<td>122.1±10.1</td>
<td>50.8±2.8</td>
<td>60.8±8.0</td>
<td>50.9±2.6</td>
<td>33.0±4.4</td>
<td>75</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>AS (male)</td>
<td>6</td>
<td>67.7±1.4</td>
<td>128.3±7.0</td>
<td>43.2±5.0*</td>
<td>44.2±7.6</td>
<td>60.8±5.6</td>
<td>23.7±4.6</td>
<td>100</td>
<td>67</td>
<td>50</td>
</tr>
</tbody>
</table>

n indicates No. of patients; BP, blood pressure, Δp mean, mean pressure gradient at aortic valve; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic dimension; FS, fractional shortening; ACEI, ACE inhibitor; ARB, angiotensin receptor blocker; and AS, aortic stenosis.

*P<0.05.

this phenomenon in the human heart have not yet been analyzed. The studies on sex differences in hypertrophy development in aortic stenosis also have not analyzed cardiac sexual hormone receptor expression or regulation. Therefore, we studied the expression and regulation of ERs in human patients with aortic stenosis together with hypertrophy-associated genes.

Methods

Patients

Left ventricular myocardial samples from 17 control subjects (8 women aged 57.4±2.1 years and 9 men aged 55.0±2.5 years) and from 14 patients with aortic valve stenosis (8 women and 6 men; Table 1) were analyzed for ER-α and -β mRNA content. In a second series, ER-α protein, calcineurin A-β, and BNP mRNA were measured in left ventricular myocardial samples of 10 control subjects (5 women and 5 men) and 11 patients with aortic stenosis (6 women and 5 men) in a largely overlapping cohort. All women in the study were postmenopausal, and hormone replacement therapy was used in only 2. The biopsies of patients with aortic stenosis were obtained from the left ventricular septum at elective aortic valve replacement surgery. The control group was composed of tissue samples of donor hearts that were rejected for logistic reasons with normal systolic cardiac function, no history of cardiac disease, and normal postmortem histology. All samples were obtained after written informed consent had been given. The study followed the rules of the Declaration of Helsinki.

Real-Time Reverse Transcription–Polymerase Chain Reaction

RNA was extracted and reverse transcribed after complete DNA digestion as described. A “hot start” real-time reverse transcription–polymerase chain reaction (RT-PCR) procedure was performed in duplicate with the Light Cycler instrument (Roche) for ER-α and -β with the use of highly specific, paired hybridization probes (Table 2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified with the use of SYBR green (Table 2).

For the calcineurin A-β and BNP measurement, a “hot start” real-time RT-PCR procedure was performed in triplicate with the Taqman instrument (ABI) with the use of SYBR green (Table 2); mRNA content of each target gene was normalized to the mRNA content of GAPDH (Table 2) in the same sample.

Immunoblotting

Left ventricular myocardial tissue samples were homogenized in a FastPrep homogenizer (FP120, Qbiogene) containing LysisMatrix particles and a double-detergent lysis buffer. After centrifugation, protein content was measured (BCA protein assay, Pierce). Myocardial proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Polyclonal antibodies directed against the C-terminus of ER-α (MC-20, SantaCruz) or monoclonal antibodies directed against GAPDH (Chemicon) were used. The signal was

TABLE 2. Sequences of Primers and Hybridization Probes Used

<table>
<thead>
<tr>
<th>Identification</th>
<th>Sequence</th>
<th>Amplicon Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>LightCycler primers and hybridization probes</td>
<td><img src="http://circ.ahajournals.org/content/images/sequence" alt="Sequence" /></td>
<td><img src="http://circ.ahajournals.org/content/images/sequence" alt="Sequence" /></td>
</tr>
<tr>
<td>ER-α forward</td>
<td>5'-GGA ATG ATG AAA GGT GGG ATA-3’</td>
<td>240</td>
</tr>
<tr>
<td>ER-α reverse</td>
<td>5'-TCT GGT AGG ATC ATA CTC GGA-3’</td>
<td></td>
</tr>
<tr>
<td>ER-α fluorescein</td>
<td>5'-CGA CCA GAT GGT CAT TGG CTT GT X</td>
<td></td>
</tr>
<tr>
<td>ER-α LCreld640</td>
<td>5'-LCred640-GGA TCG TGA GCC CCC GAT ACT CTA p</td>
<td></td>
</tr>
<tr>
<td>ER-β forward</td>
<td>5'-AAC ATC GCT AGA ACA CAC CCT A-3’</td>
<td>182</td>
</tr>
<tr>
<td>ER-β reverse</td>
<td>5'-GCC TTA CAT CCT TCA CAC GA-3’</td>
<td></td>
</tr>
<tr>
<td>ER-β fluorescein</td>
<td>5'-CAG CCC TGG TAC TGG TCC AGG TTC X</td>
<td></td>
</tr>
<tr>
<td>ER-β LCreld640</td>
<td>5'-LCred640-AAG AGG GAT GCT CAC TTC TGC GC p</td>
<td></td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>5'-CAC CAT CTT CCA GGA GCG AG-3’</td>
<td>235</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>5'-GCA GGA GCC ATT GCT GAT-3’</td>
<td></td>
</tr>
<tr>
<td>GeneAmp5700 primers</td>
<td><img src="http://circ.ahajournals.org/content/images/sequence" alt="Sequence" /></td>
<td><img src="http://circ.ahajournals.org/content/images/sequence" alt="Sequence" /></td>
</tr>
<tr>
<td>BNP forward</td>
<td>5'-ACG TCC GGG TTA CAG GAG C-3’</td>
<td>164</td>
</tr>
<tr>
<td>BNP reverse</td>
<td>5'-ACC ATG TTA CGG TGC CC-3’</td>
<td></td>
</tr>
<tr>
<td>CnA-β forward</td>
<td>5'-AAT GAG GGT GCT GCC ATC C-3’</td>
<td>175</td>
</tr>
<tr>
<td>CnA-β reverse</td>
<td>5'-AAC CTC TGT CCA CAT AAT CGC C-3’</td>
<td></td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>5'-GAA GGT GAA GGT CGT AGT C-3’</td>
<td>226</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>5'-GAA GAT GGT GAT GAT GAT TC-3’</td>
<td></td>
</tr>
</tbody>
</table>

CnA indicates calcineurin A.
visualized with the ECL detection kit (Amersham Pharmacia Biotech) and quantified with AlphaEaseFC (software, version 3.1.2, Alpha Innotech Corporation).

Immunohistochemistry
Paraffin-embedded human myocardial left ventricular samples were cut into 5-μm sections and mounted on SuperFrost glass slides. Sections were then incubated overnight at room temperature with the primary antibodies polyclonal rabbit anti–ER-α (Santa Cruz, MC-20), polyclonal rabbit anti–ER-β (Affinity BioReagents, PA1-311), monoclonal mouse anti-vimentin (Oncogene, Ab-1), and monoclonal mouse anti–troponin-T (Neomarkers, AB13-11). After they were washed, sections were incubated with secondary FITC-conjugated anti-rabbit immunoglobulins (Dianova) and Cy3-conjugated anti-mouse immunoglobulins (Dianova), washed again, and stained with DAPI (236276, Roche). Sections were mounted with Vectashield (Linaris) and viewed on the confocal microscope (Zeiss Confocal LSM510 Microscope). To control for false-positive staining for ER-α, all staining was negative (Figure 2F). Second, ER-β was localized to cardiomyocytes in left ventricular sections of control hearts. The signal was found in a striated pattern in the cytosol and in the nuclei (Figure 2A to 2D, 2G).

Statistical Analysis
Results are expressed as mean±SEM. To obtain normal distribution, mRNA expression data were logarithmically transformed and analyzed by 2-factorial ANOVA (gender, diagnosis) with interaction. Protein content was analyzed nonparametrically (Mann-Whitney U test). In subgroup analyses within controls, multiple testing was adjusted for by Bonferroni correction with factor 2. Pearson correlation coefficient was determined for logarithmically transformed data.

The level of significance was 0.05 (2 sided) in all statistical tests. All analyses were performed with the use of SPSS for Windows (release 11.5).

On the basis of sample size and SDs of this study, the effects detectable with 80% statistical power were determined.

Results
Increase in ER-α in Patients With Aortic Stenosis
ER-α mRNA was increased 2.6-fold (P=0.003) and ER-α protein content was increased 1.7-fold (P=0.026) in all patients with aortic stenosis in comparison to all controls (Figure 1A and 1B, 2I). ER-α mRNA and protein content in control hearts and their increase in patients with aortic stenosis were comparable in women and men (Figure 1C and 1D).

Increase in ER-β in Patients With Aortic Stenosis
ER-β mRNA content was increased 2.6-fold in all patients with aortic stenosis in comparison to all controls (P<0.0001) (Figure 3A). ER-β mRNA content was significantly different in female and male control hearts (P<0.005 after Bonferroni correction) (Figure 3B). Moreover, the percent increase in ER-β mRNA was more pronounced in female than in male aortic stenosis hearts (404±47% and 183±28% of sex-specific controls; P=0.005) (Figure 3B).

ER-β was also found in a striated pattern in the cytoplasm and in the nuclei of myocytes (Figure 2E and 2F, 2H).

Correlation of ER mRNA With Calcineurin A-β and BNP mRNA
Calcineurin A-β and BNP mRNA were increased in patients with aortic stenosis 2.5-fold and 10.1-fold (P<0.001, P<0.05, respectively; Figure 4A and 4B). ER-β mRNA content was inversely correlated to calcineurin A-β mRNA (r = −0.83, P=0.002) (Figure 4C) but was not correlated to BNP mRNA (Figure 4D). ER-α mRNA was correlated neither to calcineurin A-β mRNA nor to BNP mRNA (data not shown).

Effect of Medication and Hormonal Status
Eighty-six percent of the patients with aortic stenosis were treated with a diuretic, 57% received an ACE inhibitor or angiotensin receptor blocker, mainly for concomitant hypertension, and 50% were treated with a β-blocker. No signifi-
cant difference in medication was observed between female and male patients. No differences in levels of ER-α mRNA and protein and ER-β mRNA were found in patients untreated or treated with a diuretic, ACE inhibitor/angiotensin receptor blocker, or β-blocker. All women in the study were postmenopausal, and HRT was used by only 2 patients with ER expression close to the median of the group. Therefore, we cannot comment on the effect of sexual hormones on myocardial ER expression.

Discussion
We describe for the first time that ER-α and -β mRNA and ER-α protein are increased in hearts of patients with aortic stenosis. A significant inverse correlation was found between

Figure 2. A to D, Detection of ER-α in 5-μm paraffin sections of left ventricle of a control human heart by immunofluorescent staining and confocal laser-scanning microscopy. A to C show the same section stained for ER-α (FITC-green) (A); ER-α (FITC-green) and troponin T (Cy3-red) (B); and ER-α (FITC-green), troponin T (Cy3-red), and DAPI (blue) (C). B and C are merged images. Arrow indicates a nucleus. D, Negative control: myocardial section stained with antibodies directed against troponin T (Cy3-red) and ER-α (FITC-green) incubated with its blocking peptide. No nonspecific binding of primary or secondary antibody was detected for ER-α. E, F, Detection of ER-β in 5-μm paraffin sections of left ventricle of a control human heart by immunofluorescent staining and confocal laser-scanning microscopy. E and F show serial sections stained for ER-β (FITC-green) and vimentin (Cy3-red) (merged image) (E) and negative control (without primary antibodies) (F). Autofluorescence (lipofuscin) is shown in red. G, ER-α nuclear staining. Autofluorescence (lipofuscin) is shown in red. H, ER-β nuclear staining. I, Representative immunoblot. ER-α protein is upregulated in left ventricular myocardium of patients with aortic stenosis (1+2, controls; 3+4, aortic stenosis).
ER-β and calcineurin A-β mRNA. ER-α and -β protein have been visualized for the first time in adult human myocardium. Thus far, ERs have been analyzed in detail only in the rodent heart. In rat cardiomyocytes, immunofluorescence showed the ER localized in a striated pattern in the cytoplasm and estrogen-dependent nuclear translocalization of ER-α and -β. In agreement with this study, we found ER-α and -β expression in a striated pattern in the cytoplasm alternating with troponin T staining in a manner that suggests localization to the Z-disk region (Figure 2). ER-α and -β were present in some but not all nuclei, excluding the nucleoli, which may reflect inhomogeneous stimulation of myocytes by estrogen. Thus far, sex differences in ER expression in female and male rodent, primate, or human hearts have not yet been found. We found sex differences in ER-β expression in controls and a 2.5-fold greater ER-β increase with aortic stenosis in female hearts. From the statistical point of view, our sample size was only moderate. For ER-β, the power of the study would have allowed us to detect sex ratios of 1.9 within controls and 2.5 for the increase in aortic stenosis (based on logarithmically transformed data; see Methods). However, we observed significant sex ratios of 2.2 (within controls) and 2.5 (-fold increase in aortic stenosis patients; Figure 3B), suggesting that the differences in ER-β expression and greater increase with aortic stenosis in women correspond to a true phenomenon. In contrast, for ER-α mRNA, the study had power to detect sex ratios of 1.9 in the control group and 3.9 for the increase in female/male aortic stenosis patients. Because we observed sex ratios of only 1.2 (for controls and increase in aortic stenosis), our data do not suggest sex differences in the expression of ER-α, but we cannot exclude that differences might be proven significant when larger cohorts are investigated.

The isoform calcineurin A-β of the phosphatase calcineurin is particularly relevant for the development of cardiac hypertrophy because isolated knockout of this gene prevents the development of a hypertrophic response. We observed an increase in the expression of calcineurin A-β, as expected from previous studies, and found a strong inverse correlation between the ER-β and calcineurin A-β mRNA expression and greater increase with aortic stenosis in women (r=−0.83, P=0.002) in women (c) and men (e). D, ER-β mRNA was not correlated with BNP expression in aortic stenosis in women (c) and men (e). Con indicates control.
content in aortic stenosis. This suggests an association between the increase in ER-β and the suppression of the hypertrophic mediator calcineurin by as yet unknown mechanisms. Regulation of calcineurin as well as of ER-α by peptide growth factors has been described and may serve as a starting point for further investigations. 19,20

Because a relation between ER expression and markers of hypertrophy such as natriuretic peptides has been reported in the literature, 21 we investigated the correlation between ER and BNP expression. For the first time, we demonstrated that myocardial BNP expression was upregulated in human aortic stenosis. However, the ventricular BNP gene expression did not correlate with ER-α or -β mRNA. The regulation of ANP and its correlation with ER gene expression was determined in a smaller number of samples and yielded a comparable upregulation and a lack of correlation with ERs (data not shown). Therefore, thus far our findings do not support a link between the regulation of ER and ventricular natriuretic peptide gene expression.

In conclusion, we showed in this first investigation in human subjects that ER-α and -β in the human heart are upregulated by pressure overload. Further investigations in animal models are needed to evaluate this effect in detail and to determine whether sex-specific mechanisms and/or links to prohypertrophic or antihypertrophic pathways are involved.

Acknowledgments
This study was supported by the Deutsche Forschungsgemeinschaft (GRK 754). We thank Britta Hannack and Jenny Thomas for excellent laboratory work and Noel Bairey Merz for fruitful discussions of the scientific content. We also thank Rudolf Meyer for morphological evaluation of the myocardial biopsies and Elisabeth Ehler for comments on confocal laser microscopy.

References
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Circulation. 2004;110:3270-3275; originally published online November 8, 2004;
doi: 10.1161/01.CIR.0000147610.41984.E8
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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