Ligand-dependent activation of ERβ lowers blood pressure and attenuates cardiac hypertrophy in ovariectomized spontaneously hypertensive rats

Virginija Jazbutyte1, Paula Anahi Arias-Loza1, Kai Hu1, Julian Widder1, Vijayakumar Govindaraj1, Christine von Poser-Klein1, Johann Bauersachs1, Karl-Heinrich Fritzemeier2, Christa Hegele-Hartung2, Ludwig Neyses3, Georg Ertl1, and Theo Pelzer1*

1Department of Medicine, University of Würzburg, Josef-Schneider Str. 2, D-97080 Würzburg, Germany; 2Schering AG, Berlin, Germany; and 3Division of Cardiology, University of Manchester, Manchester, UK

Received 22 March 2007; revised 20 November 2007; accepted 21 November 2007; online publish-ahead-of-print 4 December 2007

KEYWORDS
oestrogen receptor; myocardium; hypertrophy; hypertension

Aims The biological effects of oestrogens are mediated by two different oestrogen receptor (ER) subtypes, ERα and ERβ, which might play different, redundant, or opposing roles in cardiovascular disease. Previously, we have shown that the selective ERα agonist 16α-L2 improves vascular relaxation, attenuates cardiac hypertrophy, and increases cardiac output without lowering elevated blood pressure in spontaneously hypertensive rats (SHR). Because ERβ-deficient mice exhibit elevated blood pressure and since the ERβ agonist 8β-VE2 attenuated hypertension in aldosterone-salt-treated rats, we have now tested the hypothesis that the isotype-selective ERβ agonist 8β-VE2 might be capable of lowering elevated blood pressure in ovariectomized SHR.

Methods and results Treatment of ovariectomized SHR with 8β-VE2 for 12 weeks conferred no uterotrophic effects but lowered elevated systolic blood pressure (−38 ± 5 mmHg, n = 31, P < 0.001 vs. placebo) as well as peripheral vascular resistance (−31.3 ± 4.6%, P < 0.001 vs. placebo). 8β-VE2 enhanced aortic ERβ expression (+75.7 ± 7.1%, P < 0.01 vs. placebo), improved NO-dependent vaso-relaxation, augmented phosphorylation of the vasodilator-stimulated phosphoprotein in isolated aortic rings (P < 0.05 vs. placebo), increased cardiac output (+20.4 ± 2.5%, P < 0.01 vs. placebo), and attenuated cardiac hypertrophy (−22.2 ± 3.2%, P < 0.01 vs. placebo). 8β-VE2, in contrast to oestradiol, did not enhance cardiac α-myosin heavy chain expression.

Conclusion Ligand-dependent activation of ERβ confers blood pressure lowering effects in SHR that are superior to those of 17β-estradiol or the ERα agonist 16α-L2 and attenuates cardiac hypertrophy primarily by a reduction of cardiac afterload without promoting uterine growth.

1. Introduction

Oestrogen effects in the cardiovascular system are mediated by two different oestrogen receptor (ER) subtypes, ERα and ERβ, which are encoded by different genes, possess a similar domain structure, and are activated by the non-selective ER agonist 17β-estradiol in a variety of cell types, including cardiac myocytes and vascular cells.1–4 The observation of uterus atrophy in ERα but not in ERβ knock-out mice as well as elevated blood pressure in ERβ but not in ERα-deficient mice clearly indicates that both receptors play different functional roles in different tissues, which promoted the development of subtype selective ER agonists as an alternative to non-selective ER ligands such as 17β-estradiol.5–9 The ERα agonist 16α-L2 and the ERβ agonist 8β-VE2 were designed based on high-resolution modelling of the ER ligand binding pocket of ERα and ERβ, respectively.9 Both compounds act as highly selective and potent ER agonists over a broad range of different ligand concentrations.10 Isotype selective ER agonists thus represent a novel tool to study the ligand-dependent function of ERα and ERβ in a variety of species and thereby complement genetic mouse models lacking functional ERα or ERβ. As we have shown previously, ligand-dependent activation of ERα improves endothelial dysfunction, augments cardiac output, and attenuates cardiac hypertrophy without lowering elevated blood pressure of ovariectomized spontaneously hypertensive rats (SHR).11,12 In contrast to SHR, hypertension in aldosterone-salt-treated rats (AST) responded to treatment with the ERα agonist 16α-L2, the ERβ agonist 8β-VE2, and the non-selective ER agonist...
17β-estradiol, these observations clearly indicate that the potency of the ERα selective agonist 16α-LE2 and of 17β-estradiol to lower blood pressure varies among different models of hypertension. Therefore, we speculated that the selective activation of ERβ might confer different effects than of ERα in SHR. To test this hypothesis, we analysed blood pressure, cardiac and vascular function, as well as gene expression in ovariectomized SHR treated with the ERβ ligand 8β-VE2.

2. Methods

2.1 Animal model and treatment

Female SHR were obtained from Charles River (IFFA CREDO, Lyon, France). The animals were ovariectomized (ovx) or sham operated at 6 weeks of age and randomized to the following treatment groups: (a) sham, (b) ovx + placebo, (c) ovx + 17β-estradiol (E2; 2 μg/kg/d, Sigma), or (d) ovx + ERβ agonist 8β-VE2 (30 μg/kg/d, Schering AG). Each group consisted of at least 10 animals. Oestriol and 8β-VE2 were dissolved in EtOH and injected subcutaneously everyday throughout the entire study using peanut oil as the carrier; ovx control animals received EtOH/peanut oil alone. The dosages of 17β-estradiol and of 8β-VE2 were chosen based on published in vivo studies to achieve physiological serum hormone levels (E2) or full activation of ERβ without causing co-activation of ERα (8β-VE2). Treatment started at 6 weeks of age and continued until haemodynamic analysis, which was performed at 18 weeks of age. Body weight, heart weight, uterus weight, and tibia length were measured following haemodynamic analysis; heart weight was normalized to tibia length to calculate relative heart weight. Serum 17β-oestradiol, angiotensin II (Ang II), and endothelin-1 (ET-1) levels were measured by radio immunoassays according to the manufacturers instructions from serum samples obtained following haemodynamic analyses (E2: DPC-Biermann; Ang II and ET-1: Peninsula). The study was conducted according to the current NIH guidelines on the care and use of laboratory animals.

2.2 Haemodynamic analysis

Haemodynamic measurements were performed after 3 months of continuous treatment under light isoflurane anaesthesia and spontaneous respiration (isoflurane 1.5vol% supplemented by 0.5l/min oxygen/min). Left ventricular (LV) pressure curves were recorded after catheter placement in the LV cavity, systolic and diastolic blood pressure measurements were obtained upon catheter withdrawal in the thoracic aorta. An electromagnetic flow probe (2.5 mm ID; Statham, Inc.) was placed around the descending aorta for continuous measurement of aortic blood flow (cardiac output). After the haemodynamic measurements, animals were euthanized by an overdose of isoflurane under continuous ECG recording. Tissue harvest was initiated after obtaining complete flatline ECGs.

2.3 Vascular and cardiac gene expression

The expression of ERα (ER21/1:2.000), ERβ (CO 1531, 1:1.000; both are generous gifts of G. Greene Univ. of Chicago), vasodilator-stimulated phosphoprotein (VASP; rabbit anti-total VASP, M4, 1.3.000, generous gift of U. Walter, Wuerzburg), and P-VASP (mouse anti-phospho-Ser1179 VASP, 16C2, 2 μg/ml, U. Walter) was analysed using western blots produced from proteins separated and transferred from crude cardiac or aortic extracts (20 μg/lane) and labelled with the indicated primary antibodies according to published techniques. VASP expression was measured as it serves as an established bio-marker of the integrity of the NO/cGMP axis. Equal gel loading was verified by Ponceau staining or western blots for GAPDH (Chemicon, 1:3.000). Absolute ERα and ERβ content was assessed from linear standard curves by blotting individual protein samples together with defined and increasing amounts of recombinant ERα and ERβ protein followed by densitometric band analysis. Cardiac expression of α- and β-myosin heavy chains (MHC) was analysed by silver staining of denaturing acrylamide gels of crude cardiac extracts. Band intensities were determined by densitometric quantification (‘ScanPack-3.0’, Biometra).

2.4 Immunohistochemical analysis

Sections from the descending aorta, the left ventricle, and from mesenteric arteries were collected in 1 M KCl, fixed in Tissue-TEC OCT, and frozen at −80°C. Two micrometre cryosections were used for immunohistochemistry for ERα (ER21, 1:100) and ERβ (CO1531, 1:100) using commercial kits (Vector Laboratories) and DAB as chromogenic substrate according to manufacturer’s instructions. Primary antibodies were omitted as well as substituted with irrelevant antibodies in control sections.

2.5 Vascular reactivity studies

Segments of 10 mm length from the descending aorta were cut into rings of 3 mm length for isometric force measurements as described previously. Aortic rings were equilibrated for 30 min under a resting tension of 2 g in oxygenated (95% O2; 5% CO2) Krebs-Henseleit solution followed by repeated contraction in KCl (100 mM/L). The resting tension of 2 g was chosen because previous studies indicated that this is the optimal resting tension for maximum force generation in aortic rings of young SHR rats. The relaxant response to cumulative doses of acetylcholine was analysed after pre-treatment with 50 mM/L KCl. A 50 mM KCl solution was chosen because it induces a submaximal pre-contraction that allowed us to study endothelium-dependent relaxation. The relaxation of each individual aortic ring was normalized to its pre-contraction level; measurements thus indicate per cent change relative to pre-contraction. Submaximal (50 mM/L) and maximal (100 mM/L) KCl-induced contractions were not different among all groups. Basal NO formation was assessed by measuring the contraction induced by a 45 min incubation with the NO-synthase inhibitor Nω-nitro-L-arginine (L-NA, 100 μmol/L) in ring segments pre-constricted with phenylephrine to about 10% of maximum contraction. Endothelium-independent relaxation was assessed using sodium nitroprusside (SNP).

2.6 Statistics

Statistical significance was calculated by one-way ANOVA, followed by standard Newman-Keuls post hoc testing using SigmaStat (Version 2.03). Correlations were determined by Spearman rank order tests. Values are given as mean ± SEM, P-values of <0.05 were considered significant.

3. Results

3.1 Global parameters

Serum oestradiol levels were low in ovariectomized SHR treated with placebo or 8β-VE2 compared with physiological levels in intact or in oestradiol-treated rats (Table 1). Serum All levels were lower in ovariectomized compared with intact rats and not altered by hormone treatment whereas endothelin I levels did not differ among all treatment groups. Body weight was elevated in oestrogen-depleted compared with sham-operated rats and decreased in 17β-estradiol but not in 8β-VE2-treated SHR. Uterus atrophy was evident in ovariectomized rats receiving placebo or 8β-VE2 but not in animals supplemented with 17β-estradiol. Absolute heart weight, which was higher in ovariectomized compared with sham-operated animals, decreased in SHR treated with 17β-estradiol or 8β-VE2.
Table 1 Global and haemodynamic parameters

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Ovx</th>
<th>Ovx + E2</th>
<th>Ovx + 8β-VE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>19</td>
<td>31</td>
</tr>
<tr>
<td>Morphometry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>203 ± 0.7</td>
<td>243 ± 0.7 * †</td>
<td>214 ± 0.2</td>
<td>240 ± 0.9 * †</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>1287 ± 133</td>
<td>1489 ± 47 * † †</td>
<td>1160 ± 36</td>
<td>1158 ± 24</td>
</tr>
<tr>
<td>Tibia length (mm)</td>
<td>34.4 ± 2</td>
<td>35.8 ± 2</td>
<td>35.0 ± 2</td>
<td>36.6 ± 3</td>
</tr>
<tr>
<td>Heart weight/tibia length (mg/mm)</td>
<td>37.4 ± 1.5</td>
<td>41.3 ± 1.4 *</td>
<td>33.1 ± 1.6 * †</td>
<td>31.6 ± 0.6 * †</td>
</tr>
<tr>
<td>Uterus weight (mg)</td>
<td>288 ± 16 †</td>
<td>92 ± 12 * † †</td>
<td>173 ± 17</td>
<td>54 ± 8 †</td>
</tr>
<tr>
<td>Hormone measurements</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum E2 (pg/ml)</td>
<td>74.9 ± 6</td>
<td>24.7 ± 1.5 * †</td>
<td>87.1 ± 6</td>
<td>21.2 ± 1.8 * †</td>
</tr>
<tr>
<td>Serum All (pg/100 μl)</td>
<td>48.5 ± 5.1</td>
<td>33.8 ± 4.1 *</td>
<td>29.1 ± 3.9 *</td>
<td>30.4 ± 2.3 *</td>
</tr>
<tr>
<td>Serum endothelin I (pg/100 μl)</td>
<td>68.2 ± 5.3</td>
<td>58.1 ± 4.4</td>
<td>59.3 ± 4.1</td>
<td>53.5 ± 3.6</td>
</tr>
<tr>
<td>Haemodynamic data</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (b.p.m.)</td>
<td>341 ± 5</td>
<td>354 ± 7</td>
<td>359 ± 8</td>
<td>337 ± 7</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>224 ± 5</td>
<td>222 ± 5</td>
<td>211 ± 5</td>
<td>184 ± 3 * † †</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>150 ± 6</td>
<td>146 ± 5</td>
<td>140 ± 5</td>
<td>129 ± 5 * †</td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td>175 ± 5</td>
<td>172 ± 5</td>
<td>160 ± 6</td>
<td>147 ± 4 *</td>
</tr>
<tr>
<td>LVdP (mmHg)</td>
<td>6.0 ± 0.5</td>
<td>5.3 ± 0.5</td>
<td>3.8 ± 0.8</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>dp/dt max (mmHg/s)</td>
<td>14 025 ± 672</td>
<td>13 671 ± 615</td>
<td>14 047 ± 755</td>
<td>12 348 ± 747</td>
</tr>
<tr>
<td>dp/dt min (mmHg/s)</td>
<td>10 550 ± 454</td>
<td>11 806 ± 573</td>
<td>12 870 ± 492</td>
<td>11 713 ± 496</td>
</tr>
<tr>
<td>Cardiac output (ml/min)</td>
<td>56 ± 2</td>
<td>54 ± 2</td>
<td>61 ± 2 *</td>
<td>65 ± 1 *</td>
</tr>
<tr>
<td>Stroke volume (μl)</td>
<td>168 ± 5</td>
<td>152 ± 3</td>
<td>171 ± 7</td>
<td>198 ± 7 *</td>
</tr>
<tr>
<td>Peripheral resistance (arbitrary units)</td>
<td>3.0 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>2.6 ± 0.1 *</td>
<td>2.2 ± 0.1 * †</td>
</tr>
</tbody>
</table>

All measurements were performed 12 weeks after ovariectomy/sham OP.

* P < 0.05 vs. sham.
† P < 0.05 vs. ovx + E2.
‡ P < 0.05 vs. ovx + 8β-VE2.
§ P < 0.05 vs. ovx.

Relative heart weight normalized to tibia length was significantly lower in oestrogen-depleted SHR receiving oestradiol or 8β-VE2 compared with placebo-treated rats.

### 3.2 Haemodynamic analysis

Systolic, diastolic, and mean arterial blood pressure levels as well as peripheral vascular resistance were significantly lower in SHR receiving the ERβ agonist 8β-VE2 compared with intact, ovariectomized, and oestradiol-treated animals (Table 1). 17β-oestradiol supplementation had only moderate and insignificant effects on blood pressure and vascular resistance. Cardiac output and LV stroke volume were 22.2 ± 5.6% (P < 0.001) and 30.3 ± 7.3% (P < 0.001) higher in SHR treated with 8β-VE2 compared with oestrogen-depleted rats. 17β-oestradiol was less efficient to improve functional cardiac parameters in ovariectomized SHR. Cardiac output correlated closely with systemic vascular resistance (r² 0.51, P < 0.001) and LV stroke volume (r² 0.68, P < 0.001) as shown in Figure 1.

### 3.3 Vascular reactivity studies

Acetylcholine induced a concentration-dependent relaxation in pre-constricted aortic rings, which was substantially diminished in rings obtained from ovariectomized compared with sham-operated SHR (Figure 2A). Endothelium-independent relaxation by SNP was not different among all groups (Figure 2B). 17β-oestradiol as well as 8β-VE2 improved the acetylcholine-induced relaxation of aortic rings from ovariectomized rats (Figure 1A; P < 0.001 for sham, ovx E2 and ovx 8β-VE2 vs. ovx placebo) without affecting the response to SNP (Figure 2B). The inhibition of NO synthase by Nω-nitro-1-arginine induced a significantly higher contraction in sham-operated than in ovariectomized animals (Figure 2C), indicating lower levels of basal NO formation in the aortae of ovariectomized SHR. Treatment of oestrogen-depleted SHR with 17β-oestradiol and 8β-VE2 augmented the L-NA-induced contraction of aortic rings.

### 3.4 Cardiac and vascular ERα and ERβ expression

Relative and absolute expression levels of ERα were uniform among cardiac and aortic extracts from all treatment groups (Figure 3A–D). ERβ expression, which was higher in cardiac compared with aortic extracts among sham-operated and ovariectomized rats receiving placebo or 17β-oestradiol, increased significantly in the aortae of SHR treated with 8β-VE2 (Figure 3A–D; * P < 0.01 ovx 8β-VE2 vs. ovx placebo or vs. ovx E2, n = 10 animals/group). Aortic extracts from intact and from ovariectomized rats treated with placebo or 17β-oestradiol contained only slightly more ERβ than ERα protein (P > 0.05) but ERβ was more abundant and thus the predominant ER subtype in the aortae of SHR treated with 8β-VE2 (Figure 3D).

The cellular expression pattern of ERα and of ERβ in cardiac sections revealed a similar staining pattern for both ER subtypes in cardiac myocytes (Figure 4). In the aorta as well as in coronary and mesenteric arteries, ERα and ERβ both localized to vascular smooth muscle cells (VSMCs) of the media and endothelial cells (ECs) of the intima layer. ERβ staining was more intense in ECs of the intima compared with adjacent VSMCs in aortic and mesenteric artery sections. No staining was detected with either secondary antibody alone or an irrelevant primary antibody (rabbit IgG, not shown).
3.5 Cardiac and vascular gene expression

Aortic VASP protein expression was comparable among all animals but VASP phosphorylation at serine 239, which serves as a sensitive marker for the functionality of the NO/cGMP axis, was lower in ovariectomized compared with sham-operated or oestrogen-depleted SHR treated with 8\(\beta\)-VE2 or 17\(\beta\)-estradiol (Figure 5A). The shift of cardiac MHC expression towards a predominant \(\beta\)-MHC expression in ovariectomized compared with intact SHR was diminished by 17\(\beta\)-estradiol supplementation but not by treatment with the ER\(\beta\) agonist 8\(\beta\)-VE2 (Figure 5B).

4. Discussion

The present study is to the best of our knowledge the first to show that ligand-dependent activation of ER\(\beta\) attenuates hypertension, vascular resistance, and cardiac hypertrophy in SHR as a widely accepted model of polygenic hypertensive heart disease.

The biological functions of oestrogens are transmitted via two different nuclear hormone receptors, ER\(\alpha\) and ER\(\beta\), which are expressed in VSMCs and in ECs of arterial origin. However, the relative abundance of both ER subtypes varies substantially between different vascular beds since ER\(\beta\) has recently been shown to be the predominant ER subtype in human internal mammary arteries.\(^\text{15}\) Non-selective ER agonists such as 17\(\beta\)-estradiol might therefore cause vasorelaxation and lower blood pressure levels via a specific ER subtype or, alternatively, via a redundant function of both receptor isoforms. The specific role of ER\(\alpha\) and ER\(\beta\), which are of pivotal importance in understanding the role of oestrogens in cardiovascular disease, has been studied in cell-based experiments and in mice harbouring targeted deletions of ER\(\alpha\) and ER\(\beta\). Although endothelial dysfunction and diminished basal NO-release have been observed in ER\(\alpha\) KO mice, ER\(\alpha\) nullizygous mice are normotensive.\(^\text{16}\) Along the same line, activation of ER\(\alpha\) improves endothelial dysfunction and enhances basal NO release but does not lower elevated blood pressure in SHR.\(^\text{12}\) But in contrast to the general hypothesis that ER\(\alpha\) might be unable to participate in blood pressure regulation, we have recently reported that the ER\(\alpha\) agonist 16\(\alpha\)-LE2 effectively lowered elevated blood pressure in female Wistar rats receiving chronic aldosterone-salt treatment.\(^\text{13}\) Together, these observations indicate that the role of ER\(\alpha\) in blood pressure maintenance is not uniform but variable and dependent on the cause of hypertension.

In contrast to mice lacking functional ER\(\alpha\) protein, systemic deletion of ER\(\beta\) results in hypertension, cardiac...
hypertrophy, and aggravates chronic heart failure and mortality in mice following experimental myocardial infarction.\textsuperscript{7,8,13,17,18} Moreover, we have recently shown that hypertension due to increased mineralocorticoid receptor activity and salt uptake is attenuated by the ER\textsuperscript{b} agonist 8\textsuperscript{b}-VE2.\textsuperscript{13} But although these observations suggest that ER\textsuperscript{b} acts as a more general and eventually also more potent modulator of hypertension, elevated blood pressure in SHR might be fully resistant to oestrogen treatment.

The present data provide solid evidence against such a general interpretation because blood pressure and peripheral vascular resistance were significantly diminished in SHR treated with the ER\textsuperscript{b} ligand 8\textsuperscript{b}-VE2. The current study thus provides novel and independent evidence for the concept of ER\textsuperscript{b} as an important modulator of blood pressure maintenance that is evident also from an association between ER\textsuperscript{b} gene polymorphism and hypertension in men.\textsuperscript{19} Interestingly, physiological doses of the non-selective

Figure 3 Cardiac and vascular ER\textsuperscript{a} and ER\textsuperscript{b} expression. ER\textsuperscript{b} was the predominant ER subtype in the aorta of SHR upon ER\textsuperscript{b} agonist treatment. (A) Representative western blot illustrating ER\textsuperscript{a} and ER\textsuperscript{b} expression in cardiac and aortic extracts (20 \textmu g/lane). (B–D) Bar graphs illustrating relative cardiac and aortic ER\textsuperscript{a} (B) and ER\textsuperscript{b} (C) expression normalized to GAPDH. (D) The bar graph illustrates absolute cardiac and aortic ER\textsuperscript{a} and ER\textsuperscript{b} content that was calculated from linear standard curves generated by blotting known and increasing amounts of recombinant ER\textsuperscript{a} and ER\textsuperscript{b} protein. Recombinant ER\textsuperscript{a} and ER\textsuperscript{b} protein (50 ng/lane) served as positive control. (*)P<0.01 ovx 8\textsuperscript{b}-VE2 vs. ovx placebo, vs. ovx E2 or vs. sham, n=10 animals/group.)
Myocardium

Coronary artery

Aorta

Mesenteric artery

<table>
<thead>
<tr>
<th></th>
<th>ERα</th>
<th>ERβ</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocardium</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Coronary artery</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Aorta</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 4 Cellular localization of ERα and ERβ receptors. Representative photomicrographs illustrate the expression pattern of ERα and ERβ in myocardial, aortic, and mesenteric artery sections. Both ER subtypes co-localized to cardiac myocytes as well as to vascular smooth muscle and endothelial cells in the aorta and in mesenteric arteries. Negative control sections, in which ERα or ERβ specific antibodies, were omitted or replaced with an irrelevant antibody, revealed no signal (control).

Figure 5 Cardiac and vascular gene expression. (A) Aortic vasodilator-stimulated phosphoprotein (VASP) expression was comparable among all groups. VASP-phosphorylation at serine239 was decreased in ovarioctomized and increased in 17β-estradiol and 8β-VE2-treated SHR (P<0.01 for sham, ovx E2 and ovx 8β-VE2 vs. ovx placebo, n = 10). (B) The α- to β-myosin heavy chain ratio was shifted towards β-MHC in ovarioctomized compared with sham-operated SHR. 17β-estradiol but not 8β-VE2 increased ratio of α- to β-MHC in oestrogen-depleted rats (P < 0.01 ovx E2 vs. ovx 8β-VE2, n = 10).

ERα and ERβ agonist 17β-estradiol conferred only minor and insignificant effects on blood pressure regulation. The pharmacological principle of 8β-VE2, which is accommodated by the ligand-binding pocket of ERβ but not of ERα, is in principal not different from ligand-dependent activation of ERβ by 17β-estradiol and thus unlikely to explain the functional difference of both ligands on blood pressure maintenance. However, the different potencies of 17β-estradiol and 8β-VE2 could be due to non-equivalent dosages of both ligands because oestradiol was supplemented to restore physiological hormone serum levels whereas the ERβ agonist was administered to achieve sub-maximal pharmacological levels at which 8β-VE2 does not yet bind and activate ERα to a measurable extent.

Alternatively, tissue- and cell type-specific expression levels of ERα and ERβ, which are a prerequisite component of oestrogen effects in the cardiovascular system, might have been different among 17β-estradiol and 8β-VE2-treated rats. Thus it is interesting to note that the application of 8β-VE2 but not of 17β-estradiol resulted in an up-regulation ERβ expression in the aortae of SHR rats. As the result, ERβ was the prominent ER isoform in the aorta in SHR receiving 8β-VE2 whereas both receptor subtypes were present at lower and approximately equal amounts in oestradiol-treated rats. Increased expression of ERβ might have therefore resulted in a stronger activation of this receptor subtype in 8β-VE2 compared with 17β-estradiol-treated SHR. In addition, one might speculate that the simultaneous activation of ERα in 17β-estradiol-treated SHR might have prevented the up-regulation of ERβ that was seen with 8β-VE2 treatment. Further studies using selective ERα antagonists should clarify this hypothesis. Further studies will also be required to determine whether gender effects, which have been described to affect cardiac hypertrophy in ERβ nullizygous mice, do also affect the efficacy of selective ER agonists in the cardiovascular system. In addition, ageing effects might attenuate the potency of 16α-LE2 and 8β-VE2 to inhibit pathological cardiac growth and hypertension since aged SHR respond very different to 17β-estradiol supplementation compared with young rats.

Local NO-bioavailability in endothelial and VSMCs is a critical regulator of vascular tone, vascular resistance, and blood pressure that has previously been linked to ERα and to ERβ activity.33,24 Improved acetylcholine-induced relaxation of intact aortic rings obtained from ovarioctomized SHR treated with 17β-estradiol or 8β-VE2 compared with placebo indicates improved NO-bioavailability. These findings might be explained by structural differences between the aortas from different treatment groups. Although comparisons of contractile forces normalized to tissue cross-sectional areas would have shed more light on this possibility, structural differences are unlikely to fully explain the current observations because submaximal (50 mmol/L) and maximal (100 mmol/L) KCl-induced absolute contractile forces were not different among the groups.

The interpretation of increased basal NO-generation in 8β-VE2-treated SHR is also evident from the enhanced contractile response of aortic rings in response to the NO-synthase inhibitor L-NA. Aortic VASP protein phosphorylation at serine239 but not VASP expression was higher in sham-operated and hormone-substituted rats compared with ovarioctomized rats receiving placebo. Enabled/vasodilator-stimulated phosphoproteins (Ena/VASP), which play an important role in actin polymerization and cell motility, are phosphorylated specifically at serine239 by cGMP kinase, whose activity depends on local nitric oxide.
availability. Therefore, enhanced VASP phosphorylation in sham operated and hormone-treated rats appears as a suitable indicator of increased local NO-bio-availability in SHR receiving 17β-estradiol or the ERβ agonist 8β-VE2.

However, these observations do not necessarily establish a causative mechanism to explain lower blood pressure in SHR because impaired vascular NO-generation is a common feature in hypertension. Accordingly, enhanced NO-availability might also be the consequence rather than the cause of lower blood pressure in ERβ agonist-treated SHR. Moreover, hypertension in SHR was not attenuated by the ERα selective agonist 16α-LE2, despite enhanced vascular NO-bioavailability, which indicates that vascular NO-generation does not necessarily translate into lower blood pressure levels in SHR. Collectively, these studies confirm the concept that both ER subtypes are capable to enhance vascular NO-generation via genomic (i.e. NOS expression) and also via non-genomic (i.e. NOS activity) mechanisms. However, the relative importance of ERβ-mediated NO-generation requires further analysis because hypertension in SHR depends on numerous factors, including increased oxidative stress, altered sodium homeostasis, genetic susceptibility and environmental imprinting, elevated mineralocorticoid levels, and to increased hypothalamic noradrenaline release.

In the present study, we could not address all of these mechanisms that may relate to the ability of 8β-VE2 to attenuate hypertension in SHR. However, neither lower serum angiotensin II content among ovariectomized SHR treated with placebo, 17β-estradiol, and 8β-VE2 compared with intact rats nor serum endothelin-1 levels, aortic angiotensin II type I, angiotensin II type-II, and ET-1A and ET-1B receptor expression explained the effect of 8β-VE2 on elevated blood pressure (P.A. Arias-Loza, unpublished observations). More strictly defined models of hypertension might thus be advantageous to identify mechanisms that are related and eventually responsible for the antihypertensive effect of 8β-VE2 in SHR. These studies might also take into account the fact that vasomotor studies in resistance arteries, which stained positive for ERα and ERβ protein, could eventually provide additional information on ERβ-mediated vasorelaxation that could not be obtained from aortic specimens.

Oestrogens attenuate the development of cardiac hypertrophy, which raises the question whether a particular ER subtype might be required or sufficient to protect the heart against pathological growth. Both ER subtypes are robustly expressed in human and in rat cardiovascular tissues whereas cardiac ER expression appears to be somewhat lower in mice. Therefore, subtype selective agonists for ERα and ERβ could in principle attenuate cardiac hypertrophy either indirectly by lowering blood pressure and hence cardiac afterload or, alternatively, via direct effects on the myocardium. As we reported previously, the ERα agonist 16α-LE2 attenuated cardiac hypertrophy in SHR most likely via such direct effects because the selective activation of ERα did not confer a blood pressure lowering effect in these rats. The present observations indicate that the activation of ERβ by 8β-VE2 attenuates cardiac hypertrophy in SHR primarily via a reduction of cardiac afterload. But we cannot formally rule out additional and direct effects of 8β-VE2 on cardiac signal transduction pathways that become activated during cardiac hypertrophy.

A relevant function of ERβ in cardiac hypertrophy is evident from recent studies showing that cardiac hypertrophy decreases upon oestrogen supplementation in ERα−/− but not in ERβ−/− mice with transverse aortic constriction. Moreover, our present observations are in good agreement with increased cardiac mass in ERβ knock-out mice that is also related to hypertension.

Cardiac hypertrophy represents an established and important risk factor for the development of chronic heart failure. Therefore, although young SHR do not exhibit signs of heart failure, it is interesting to note that cardiac output and LV stroke volume were elevated in oestradiol and in 8β-VE2-treated SHR to a similar extent than we observed previously upon treatment with the ERα agonist 16α-LE2. But in contrast to ERα activation, enhanced LV function in 8β-VE2-treated rats was closely associated with decreased vascular resistance. Impaired cardiac contractility and oestrogen depletion have frequently been associated with a shift of cardiac isomyosin composition towards predominant βMHC expression. However, it is unknown whether both ER subtypes confer redundant or specific effects in regulating cardiac MHC composition. Since cardiac αMHC expression in ovariectomized SHR responds to ERα agonist treatment in the very same way as to 17β-estradiol supplementation, it is interesting to note that the ERβ agonist 8β-VE2 did not change cardiac isomyosin composition in SHR. Unaltered cardiac MHC expression in placebo and 8β-VE2-treated SHR is thus unlikely to explain increased cardiac output upon ERβ agonist treatment. In addition, these observations indicate for the first time that non-selective ER agonists such as 17β-estradiol modulate cardiac MHC composition via the ERα but not via the ERβ receptor.

5. Conclusions
In summary, we have shown that the ERβ selective agonist 8β-VE2 confers superior effects on elevated blood pressure in female SHR rats compared with the selective ERα agonist 16α-LE2 or 17β-oestradiol. Pharmacological activation of ERβ does not stimulate uterine growth and could provide means to enhance the pharmacological safety of currently available oestrogens.

Conflict of interest: T.P. and L.N. have received financial support from the Bayer Schering Pharma AG Berlin that relates to the evaluation of novel steroid hormone receptor ligands.

Funding
Interdisciplinary Center for Clinical Research ‘IZKF’ Würzburg (Z4-70, E83-N to T.P.).

References


