Differential role of estrogen receptor isoforms in the cardiovascular system of young and senescent rats

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius- Maximilians- Universität Würzburg

vorgelegt von
Virginija Jazbutyte, MSc
aus Skuodas, Litauen

Würzburg, 2007

Eingereicht am:

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. M. Müller

Gutachter: Prof. Dr. rer. nat. K. Scheller
Gutachter: Priv. Doz. Dr. med. O. Ritter

Betreuer: Dr. med. Theo Pelzer

Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

The study was performed under supervision of Dr. med. Theo Pelzer in the laboratory of Molecular Cardiology, Medical Clinic and Policlinic I (Director Prof. Dr. G. Ertl) at Julius- Maximilians University in Würzburg, Germany. My PhD studies were supported by the German Academic Exchange Service ("DAAD", Promotionstipendium) and by the University Clinics and Policlinics in Würzburg (Ausbildungsstipendium).

Declaration

Hereby, I declare that the submitted dissertation was completed by myself and no others and that I have not used any sources or materials others than those enclosed. Moreover, I declare that the dissertation has not been submitted further in this form or any other form and has not been used for obtaining any other equivalent qualification in any other organization. Additionally, I have not applied or will attempt to apply for any other degree or qualification in relation to this work.

Würzburg, 11.05.2007

(Virginija Jazbutyte, MSc)

To my family

Content

Summary

Cardiovascular disease is the major cause of mortality morbidity in both men and women in industrialized countries. The incidence of cardiovascular diseases in pre-menopausal women is lower compared to age-matched men but the risk of heart diseases increases dramatically after the onset of menopause. Therefore, it has been postulated that female sex hormones play an important role in cardiovascular health in pre-menopausal women. In contrast to clinical data, which failed to show positive estrogen effects on cardiovascular system of post-menopausal women, extensive experimental studies indicated cardioprotective effects of estrogens in laboratory animals. The majority of experimental estrogen substitution studies were performed with young individuals, thus the effects of ageing remain neglected and are poorly understood. The present project is the first attempt to study the cardiac effects of each estrogen receptor isoform (estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ)) in adult ("menopausal") and senescent ("post- menopausal") hypertensive rats. The female senescent spontaneously hypertensive rats (SHR) served as a model system for age- associated hypertension in females whereas young individuals were used for control experiments. Young and senescent SHR rats were treated with 17β- estradiol as well as new estrogen receptor isoform selective ligands 16α-LE_2 (ERα agonist) and 8β-VE_2 (ERβ agonist). The results showed different functions of both estrogen receptor isoforms in cardiovascular system: ERα attenuated cardiac hypertrophy but not hypertension whereas ERβ could significantly reduce both, blood pressure and cardiac hypertrophy. Surprisingly, both agonists and 17β- estradiol were effective in young animals but *not* in senescent SHR rats. These findings match with the clinical data and could be related to altered estrogen metabolism in senescent rats, since estrogen plasma levels did not increase to measurable extent in senescent animals receiving estrogen. Estrogen is metabolized by several 17β- hydroxysteroid dehydrogenase isoforms. In the current study, 17β- hydroxysteroid dehydrogenase type 10 (17β-HSD10) was identified as a novel protein- protein interaction partner of estrogen receptor alpha ligand binding domain (ERαLBD) in human heart. Cellular localization experiments of ERα in the cardiac myocytes showed nuclear and cytosolic localization pattern which overlapped partially with that of cardiac

mitochondria. 17β-HSD10 is localized only in mitochondria. Direct interaction of both proteins was confirmed by pull- down experiments where 17β-HSD10 could be co-precipitated with ERα. Interestingly, protein interaction could be detected only under estrogen- free conditions whereas the presence of estrogen in the system blocked this interaction. Enzymatic assay which was developed in our laboratory, helped to define functional relevance of this interaction. The data obtained from enzymatic assays and protein- protein interaction studies strongly suggest that estrogen receptor could play an important role in the control of intracellular (or mitochondrial) estogen metabolism.

The second potential ERα interaction partner in the heart- bladder cancer associated protein 10 (BLCAP10) - was initially identified in non- invasive bladder cancer cell lines. BLCAP10 protein expression in the heart as well as its localization pattern in cardiac myocytes is shown in the last part of the theses. Due to perinuclear localization similarity with ERβ, we conclude that BLCAP10 could interact with ERβ rather than with ERα. Poor BLCAP10 protein overexpression and toxicity in both, bacteria and eukaryotic cells, suggested that BLCAP10 could be involved in cell- cycle and/ or protein expression control.

In summary, the results showed that isoform selective activation of estrogen receptors exert divergent effects in the cardiovascular system both by upregulation of αMHC expression or by lowering blood pressure. Hormones were effective in young animals but had only minor effects in senescent rats. The new ERα protein- protein interaction partners identified during the project provide new information about estrogen receptor function in the heart and its possible role in the regulation of estrogen homeostasis.

Zusammenfassung

In den Industrieländern sind kardiovaskuläre Erkrankungen die Hauptursache von Mortalität und Morbidität bei Männer und Frauen. Das Auftreten kardiovaskulärer Erkrankungen ist bei pre-menopausalen Frauen niedriger als bei Männern, doch steigt das Risiko nach der Menopause dramatisch an. Aus diesem Grund wurde postuliert, dass weibliche Sexualhormone eine wichtige Rolle bei der Prävention kardiovaskulärer Erkrankungen spielen. Obwohl in klinischen Studien für das Estrogen kein Effekt nachgewiesen wurde, ist dessen kardioprotektiver Effekt experimentell belegt. Dies mag daran liegen, dass in aller Regel für diese Experimente Jungtiere verwendet wurden. Hierdurch wurden die noch unzureichend verstandenen Auswirkungen der Alterung vermieden.

In der vorliegenden Arbeit wurden zum ersten Mal kardioprotektive Effekte der beiden Estrogenrezeptor (ER)-Isoformen ERα und ERβ in erwachsenen („menopausalen“) und seneszenten („post-menopausalen) hypertensiven Ratten nachgewiesen. Weibliche seneszente spontan-hypertensive Ratten (SHR) wurden als Modell für den Alters-assoziierten Bluthochdruck bei Frauen verwendet. Junge Ratten dienten als Kontrolle. Junge und seneszente Ratten wurden mit 17β-Estradiol und den neuen ERα- und ERβ-Agonisten 16α-LE_2 bzw. 8β-VE_2 behandelt. Beide Estrogenrezeptor-Isoformen wirkten unterschiedlich auf das kardiovaskuläre System. So minderte 16α-LE_2 die kardiale Hypertrophie, nicht aber die Hypertension, während 8β-VE_2 sowohl Hypertrophie als auch Hypertension verringerte. Beide Agonisten und 17β-Estradiol waren überraschenderweise nur in jungen, nicht aber in seneszenten Ratten effektiv. Diese Ergebnisse korrelieren mit klinischen Daten und lassen sich vermutlich mit dem geänderten Estrogen-Metabolismus älterer Ratten erklären. So waren ihre Estrogen-Plasmaspiegel nach Estrogen-Injektion nicht erhöht.

Estrogen wird durch zahlreiche Isoformen der 17β-Hydroxysteroid-Dehydrogenase metabolisiert. In dieser Arbeit wurde die Isoform Typ 10 (17β-HSD10) als bisher unbekannter Interaktionspartner für die Ligandenbindungs-Domäne des Estrogenrezeptors in menschlichen Herzen identifiziert. Der Estrogenrezeptor ERα wurde im Kern und Zytosol humaner Kardiomyozyten nachgewiesen. Zytosolisches Lokalisationsmuster deckte sich teilweise mit

dem von Herzmitochondrien. 17β- HSD10 ist ausschließlich in Mitochondrien vorhanden. Die direkte Interaktion zwischen ERα und 17β- HSD10 wurde mit *pull down*-Experimenten bestätigt, wo beide Proteine co-präzipitiert wurden. Die Interaktion von ERα und 17β- HSD10 wurde interessanterweise nur unter estrogenfreien Bedingungen nachgewiesen, während Estrogen diese Interaktion blockierte. Mit den in unserem Labor etablierten enzymatischen Untersuchungen wurde die funktionelle Bedeutung dieser Interaktion untersucht. Diese Ergebnisse sowie die Protein-Protein Interaktionsstudien lassen auf eine wichtige Rolle des Estrogen-Rezeptors ERα bei der Kontrolle des zellulären und mitochondrialen Estrogen-Metabolismus schließen.

Der zweite Interaktionspartner von ERα ist BLCAP10, das „Bladder Cancer Associated Protein 10“, das ursprünglich in Zelllinien nicht-invasiver Blasenkarzinome entdeckt wurde. Die Ergebnisse zur Expression von BLCAP10 im Herzen sowie das Lokalisationsmuster in Kardiomyozyten sind im letzten Teil der Arbeit vorgestellt. Wegen der Ähnlichkeit der perinukleären Lokalisation von BLCAP10 und ERβ erscheint eine Interaktion beider Proteine möglich. Die geringe Überexpression von BLCAP 10 in Bakterien und eukaryotischen Zellen deutet auf eine Funktion von BLCAP 10 im Zellzyklus oder bei der Proteinexpression hin.

Die Ergebnisse zeigen somit, dass eine Isoform-selektive Aktivierung von Estrogenrezeptoren divergente Effekte im kardiovaskulären System auslösen. So kommt es zu einer Hochregulierung der αMHC-Expression oder zur Senkung des Bluthochdrucks. Hormone, 17β-Estradiol bzw. die Agonisten 16α-LE_2 und 8β-VE_2, waren effektiv in jungen Tieren, zeigten aber nur geringe Effekte in alten Tieren. Der in dieser Arbeit identifizierte neue Interaktionspartner von ERα, das 17β-HSD10, liefert neue Informationen zur Funktion des Estrogenrezeptors im Herzen und zur möglichen Rolle bei der Regulierung der Estrogen-Homeostasis.

1. Introduction

In industrialized countries, coronary heart disease (CHD) is the leading cause of death in both, men and women [1, 2]. The incidence of cardiovascular disease in pre-menopausal women is lower compared to their age-matched male counterparts but with the onset of menopause, the risk to develop cardiovascular disease becomes identical in men and in women [3]. Therefore, it has been postulated that female sex hormones, especially estrogens, may play a cardioprotective role in pre-menopausal women. 17β-estradiol (E2) is synthesized by the ovaries of premenopausal women and has important physiological effects on reproductive tissues. Beyond that, estrogens preserve bone mineral density, reduce the risk of osteoporosis and lower cholesterol serum levels [4]. Numerous experimental studies have demonstrated that estrogen is a potent cardioprotective hormone, leading to the assumption that administration of estrogens may decrease the risk of cardiovascular disease in postmenopausal women. Indeed, among the groups of the postmenopausal women who have higher risk to develop cardiovascular disease, hormone therapy has been associated with improved outcomes of cardiovascular events in observational studies [5]. However, controlled clinical studies, such as the Randomized Trial of Estrogen Plus Progestin for Prevention of Coronary Heart Disease in Postmenopausal Women (HERS) [6] and the recently terminated Women's Health Initiative study [7] showed that combination of equine estrogen and medroxyprogesterone acetate (MPA) did not reduce the rate of cardiovascular events. Moreover, combined estrogen/ progesterone treatment increased the risk of thromboembolic events. These findings raise important concerns on the safety of hormone replacement therapy. During the recent past, many epidemiological and clinical studies were performed in Asian countries [8-10] as well as in the Mediterranean part of Europe [11, 12]. These studies showed positive effects of biologically active natural plant estrogens- isoflavonic phytoestrogens, or isoflavones- on blood pressure, osteoporosis and life quality in aged women. Due to geographical and/or cultural reasons, not all women can or will consume food which is rich in natural plant estrogens. Synthetic ligands that possess the beneficial estrogen effects and lack its negative side effects, so-called selective estrogen receptor modulators

(SERMs), were suggested as an alternative option to treat menopause-associated symptoms and eventually to prevent heart disease.

Although the role of estrogen in providing pharmacological cardioprotection is controversial, sex hormones do have an effect on the cardiovascular system. Despite growing number of reports in the literature identifying sex- related differences in both rodents and humans, the underlying mechanisms have yet to be determined. In experimental studies, functional estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) have been demonstrated in ventricular myocardium of male and female patients[13-15].

The reports linking hormone replacement therapies (HRT) to increased risks of cancer, cardiac disease, stroke and pulmonary emboli raise questions about the safety of HRT. Several alternatives for 17β- estradiol, such as SERMs and estrogen receptor isoform- selective ligands, could be considered as potential candidates for hormone replacement therapies. Since 17β- estradiol binds to ERα and ERβ with the similar affinity, it remains unknown whether activation of a specific estrogen receptor subtype might give similar or divergent results. Therefore, more extensive knowledge about estrogen receptor biology and structure as well as their functional studies are required to improve the safety of HRT.

1.1. Estrogen receptor structure and biology

17β-estradiol (E2) is the most potent endogenous estrogen. E2 serum levels decline in women undergoing menopause [16]. The physiological action of estrogens is mediated via two different receptors: estrogen receptor alpha and beta (ERα and ERβ, respectively). Both ERs belong to the nuclear receptor superfamily. Other steroid hormone receptors, such as the progesterone receptor (PR), the androgen receptor (AR), the retinoic acid receptor (rexinoid) (RXR), the vitamin D receptor (VDR), the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR) and a large group of so- called “orphan receptors” (their endogenous ligands are still unknown) are members of the same superfamily [17, 18]. A common feature of nuclear hormone receptors is their mode of action as ligand dependent transcription factors. Both ERs show a different tissue distribution pattern: ERα is mainly expressed in classical estrogen target tissues, such as the uterus and the mammary gland. Estrogen receptor beta has been localized in “non-classical” estradiol target tissues, such

as the central nervous system, the cardiovascular system, the immune system, the urogenital tract, the liver, the kidneys, the bones and the lung. Importantly, ERα promotes uterine growth whereas ERβ shows no uterotrophic effects [19-21]. Estrogen binding to its cognate receptor was described in the late sixties [22, 23]. The estrogen receptor alpha gene maps to chromosome 6, its exact location is 6q24- q27 [24]. The gene spans more than 322 kb region and contains 8 exons and 7 introns. Estrogen receptor alpha corresponds to a protein of 595 amino acids with the molecular weight of 67kD and consists of 6 functional domains: A, B, C, D, E, F. The N- terminal A/B domain, containing ligand- independent transcription activation function (TAF1), and the domain C (DNA-binding domain, DBD) are highly conserved among species. The DBD domain contains two subregions (CI and CII) with 2 zinc- fingers which play an essential role in binding of the estrogen receptors to estrogen response elements (ERE) in the target promoter and promote estrogen receptor dimerization. The “hinge” region (domain D) separates DBD and C- terminal region E/F which contains the ligand binding domain (LBD). Crystallographic analyses of the ERα and the ERβ ligand binding pockets revealed the determinants of ligand selectivity between the two receptor isoforms. Residues within the ligand binding pocket as well as distal secondary structural interactions contribute not only to subtype- specific positioning of the ligand but also serve as a platform for isoform- selective ligand design and synthesis [25, 26]. The ligand binding domain contains also the sites for protein- protein interactions with heat shock proteins (hsp) and immunophilins [27, 28] and a ligand- dependent transcriptional activation domain, termed transcription activation function TAF2 [29-31]. The domain F is a variable region and its functional role is not completely understood although the analysis of the F domain revealed that it is important for discrimination between agonistic and antagonistic ligands [32]. Moreover, Montano and colleagues showed that F domain can modulate the magnitude of gene transcription by estrogens and it determines the suppression effectiveness of estrogen-stimulated gene transcription by antiestrogens [33].

The existence of a second estrogen receptor isoform- estrogen receptor beta (ERβ)-as well as the isolation of cDNA clones from rat prostate and ovary cDNA libraries were reported in 1996 by Kuiper et al [34]. In the same year mouse and human ERβ were cloned [35-37]. The ERβ gene has been mapped to band q22-24 in chromosome 14. The ERβ gene contains 8 exons and 7 introns

(253.68 kb). ERβ cDNA consists of 1592 bp and encodes a 530 aa protein (M_r 59.2kD). The domain structure of ERβ is very similar to that of estrogen receptor alpha i.e. the DNA binding domain of ERβ possesses 95% homology with ERα DBD but the ligand binding domain (LBD) of ERβ shares only 53% homology with the ERα LBD. The experimental studies showed that both estrogen receptors bind 17β- estradiol with similar affinity despite low sequence identity in the LBD of both receptors. The ligand binding cavity is completely shielded from the external environment and buries the ligand in the highly hydrophobic cleft consisting of 22 hydrophobic residues. Two polar residues located on the opposite sites of the ligand binding pocket are involved in anchoring of ligand hydroxyl moieties [38]. Importantly, some synthetic or natural ligands have different downstream function and/ or relative affinities to ERα and ERβ. For example, raloxifen can bind to both, ERα and ERβ with similar affinity but completely different outcomes. In the complex with ERα, raloxifen acts as pure agonist whereas raloxifen in the complex with ERβ shows antagonistic properties. Genistein shows higher binding affinity to ERβ than to ERα [38]. The detailed protein structure of ERα and ERβ is presented in figure 1.

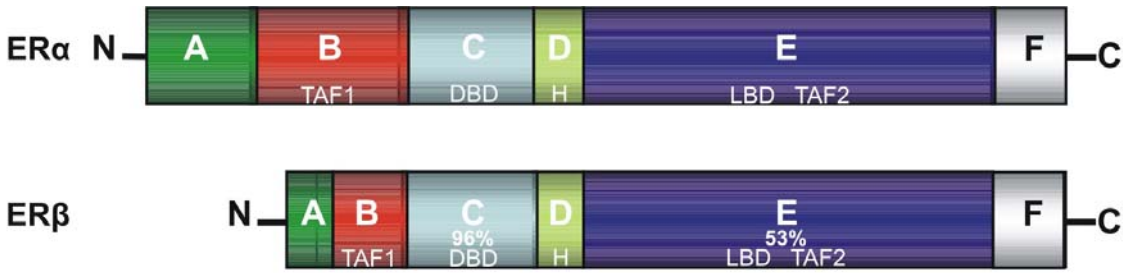


Figure 1. Domain structure of estrogen receptors alpha (ERα) and beta (ERβ). Both estrogen receptor isoforms possess similar domain structures: N- terminal domain A/B contains ligand independent transcription activation function 1 (TAF1), C domain contain highly conserved DNA- binding domain. Domain H represents a hinge region and domain E contains ligand binding domain (LBD) and ligand-dependent transcription activation function 2 (TAF2). The variable domain F is localized in estrogen receptor C- terminus.

1.2. Estrogen receptor function and regulation

Estrogen receptors are the members of a superfamily of ligand- dependent transcription factors. To date, three distinct action mechanisms of estrogen receptors have been identified: 1) genomic, “classical” action, mechanism 2)

rapid, non-genomic mechanism and 3) functional activity via protein- protein interaction.

The genomic action of activated estrogen receptor is based on its ability bind to the promoter of its target gene via two zinc finger domains. Due to lipophilic properties of steroid hormones they can freely diffuse into the cell through bilipidic cell membrane and bind to their receptors in the cytoplasm. Upon ligand binding, receptor monomers form dimers (homodimers, or heterodimers) via their dimerisation interface and the ligand-estrogen receptor complex translocates into the nucleus and attaches to estrogen receptor response element (ERE) in the promoter of estrogen receptor regulated target genes. EREs are palindromic sequences consisting of two 5 bp fragments separated by 3 bp spacer. The ERE of vitellogenin promoter was designated as “classical“ consensus sequence being GGTCANNNTGACC, where NNN are any bp. As a consequence of estrogen receptor interaction with an active ERE, target gene expression can be either activated or suppressed.

Estrogen receptors mediate not only transcriptional gene regulation (genomic action). ERs are also involved in non-genomic regulation of their target proteins via phosphorylation events. Several “non- classical” estrogen receptor action mechanisms were identified so far: induction of NO release in vascular cells [39, 40], Ras and Raf-1 kinase induction in MCF7cells [41] as well as estrogen receptor mediated rapid induction of Egr-1 (early growth response gene-1) via ERK1/2 pathway [42]. Other estrogen receptor activity regulation mechanisms involve phosphorylation events [43] and cross-talk with various signal transduction pathways, including insulin- like growth factor (IGF) signal transduction pathway [44], protein kinase A (PKA) [45, 46] as well as the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway [47, 48]. Estrogen and insulin growth factor (IGF) may thus act synergistically and both, estrogen receptor and IGF, pathways can influence each other: estrogen alters the expression levels of important members of IGF signal transduction pathway including IGF-I, IGF-II, IGF-binding proteins and IGF type I receptor (IGF-RI) in human breast cancer cells [49, 50]. Alternatively, IGF signalling can induce phosphorylation of estrogen receptor alpha [51]. Other cAMP- mediated signal transduction pathways, such as epidermal growth factor, are capable to phosphorylate and hence activate estrogen receptors even in the absence of ligand [52].

Estrogen receptor interaction with other proteins plays a key role in ER activity. The specific regulation of estrogen receptor target genes is a complex process that depends on cell and/ or tissue type, presence of estrogen receptor co-activators and co-repressors and availability of estrogen receptor ligands. The cellular context of ER activity is determined by availability of multiple proteins that interact with estrogen receptors in the cell. After direct or indirect association with estrogen receptors, cofactors influence chromatin remodelling and/or recruitment of basal transcription factors and RNA polymerase II [53-55]. Histone acetylation plays an important role in remodelling chromatin structure and facilitating transcription. Histone acetylation is a dynamic process directed by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Transcription factors recruit coactivators with HAT activity to regulatory DNA sites where they act as multimolecular complexes. These complexes either stabilize the formation of pre-initiation complexes or disrupt the chromatin structure and facilitate the access to DNA for other transcriptional regulators [56]. Transcriptional activation by ERα and ERβ involves the recruitment of several coactivators such as CBP/p300, p/CAF and the members of the SRC1 family which have been shown to possess acetyltransferase activity whereas most of ER corepressors possess HDAC activity [15, 54, 57, 58]. The cofactors regulate proper interaction of estrogen receptors with their response elements on target gene promoters and mediate protein- protein interaction of ERs with basic transcription machinery. The other group of estrogen receptor cofactors are corepressors. Transcriptional repression via ligand or estrogen receptors can occur either in the absence of the ligand or in the presence of the ligand or estrogen receptor antagonists. The repression can occur via several mechanisms: 1) via co-repressor binding directly to DNA and, thus, competition with co-activators, 2) via interference with co-activators after binding to a non-overlapping activation site (quenching) or 3) due to direct silencing of basic transcription machinery irrespective of the presence or absence of co-regulatory factors. There are only few estrogen receptor corepressors known to date: REA which is specific for liganded estrogen receptor (ligands:estrogen, tamoxifen), Ssn6/Tup1, Smad4, L7/SPA [59].

All three estrogen receptor action mechanisms take place not only in the “classical” estradiol target organs but may also affect cardiac gene expression and function. The knowledge obtained from basic estrogen receptor studies

provides information that is essential to understand the role of estrogens and estrogen receptors in the heart.

1.3. Estrogen receptor protein- protein interactions

The capability of estrogen receptor alpha and beta to interact with other proteins is mediated by different domains of the receptors including the ligand binding domains (LBDs). The LBD promotes association with a diverse group of proteins via specific LXXLL motifs (where L is leucine and X is any amino acid). The presence and function of LXXLL motif in ER- interacting proteins was described by Heery and colleagues [60]. They showed that nuclear protein binding to the estrogen receptors is dependent upon the integrity of the LXXLL motifs and on key hydrophobic residues in a conserved helix 12 of the estrogen receptor. In the absence of ligand, ERs are localized in the cytoplasm and associated with chaperones such as Hsp90. The receptor- chaperone complex dissociates in the presence of hormone and moves to the nucleus [61]. Since the ERs are transcription factors, the vast majority of receptor interaction partners are nuclear proteins including general transcription factors, sequence specific transcription factors, coactivators as well as corepressors. An overview of estrogen receptor interacting proteins is presented in figure 2.

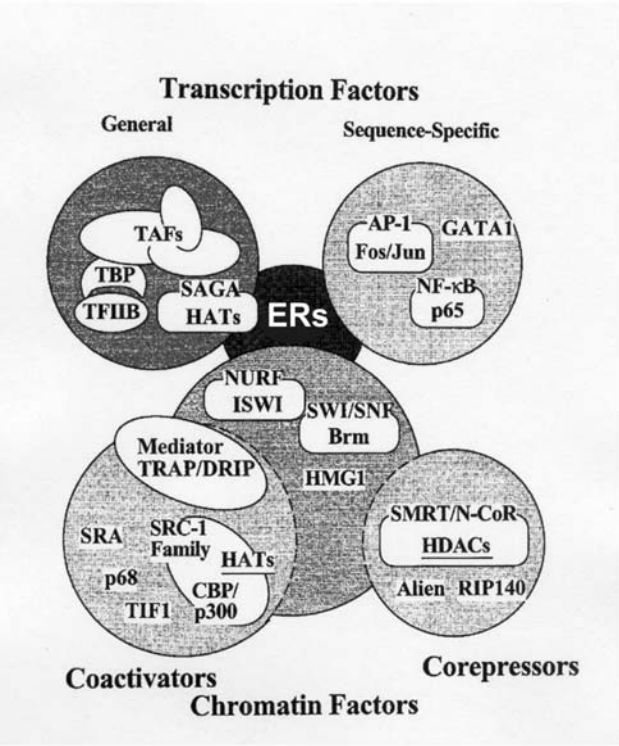


Figure 2. Overview of estrogen receptor protein- protein interaction partners in the nucleus. For each class (general transcription partners, sequence-specific transcription partners, co-activators, co- repressors and chromatin factors) typical examples are shown. (According to M. Beato and J. Klug, *Human Reproduction Update, 2000*).

Upon ligand binding to the ER, structural changes in ligand binding domain (LBD) are initiated, the essential feature is a relocation of helix H12 and subsequent interaction with CBP, p300, SRC1, P/CIP and GCN5 which form large coactivator complexes and act as histone acetyltransferases (HAT). They acetylate the specific residues in the N- terminal region of different histones, a process that plays an important role in the opening of chromatin during transcription activation. While co-activators acetylate histones, the other class of ER- interacting proteins (corepressors) recruit histone deacetylase (HDAC) activities that reverse this process. Deacetylated histones are associated with silent regions of the genome and histone acetylation and deacetylation shuffle nucleosomal targets between condensed and relaxed chromatin, the latter being requisite for transcriptional activation. In addition to the major "nuclear" receptor fraction, a small pool of ERs is localized to the plasma membrane. The studies performed in several laboratories have confirmed the ability of estrogen receptors to activate G protein-related signalling at the plasma membrane. Membrane ERs possibly exist as a cytoplasmic pool tethered to the inner face of the plasma membrane bilayer through binding to proteins such as caveolin-1 [62], in conjugation with MNAR (modulator of nongenomic activity of ER) [63, 64] , or Src and IGF-1 [65]. Beyond the nucleus, the presence of estrogen response elements (EREs) in mouse and human mitochondrial genome was first described by Sekeris in 1990 [66]. The analysis of subcellular distribution of estrogen receptor alpha and beta in rabbit uterus and ovary showed that both ER isoforms could be detected in mitochondria and microsomes of the uterus, while ERα but not ERβ was the dominant isoform in mitochondria of the ovary [67]. Further studies revealed larger amounts of ERβ than ERα in mitochondria of E2- treated MCF-7 cells [68]. Moreover, several groups could detect only ERβ but not ERα in the mitochondria of human lens epithelial cells [69] and mouse cardiac myocytes and neurons [70]. The data and statements about the presence of ERβ exclusively in mitochondria remain highly controversial. Since estrogen receptor functions in large protein complexes, further studies are required to identify estrogen receptor interaction partners in mitochondria. Besides the liver and the brain, the heart is very rich in mitochondria. Therefore, it would be interesting not only to find out whether heart mitochondria contain estrogen receptors, but also to analyse the mechanisms responsible for the improved

function of isolated estrogen treated heart mitochondria reported by Morkuniene et al. [71, 72].

1.4. Estrogen receptors in the heart

ERα and ERβ are both expressed throughout the cardiovascular system and several target genes for estrogens have meanwhile been identified, including proteins that modulate lipid clearance, vascular tone, cardiac contractility and cell proliferation. Estrogen receptors have been identified in vascular smooth muscle cells, vascular endothelial cells [73, 74], cardiac fibroblasts and cardiac myocytes [15, 75]. Long-term estrogen effects involve changes in gene and protein expression, such as L- type calcium channel, connexin43, c-fos, Egr1 as well as inducible (i)NOS and endothelial (e)NOS [76-80]. Additionally, several target genes for estrogens have been identified in the heart, including those modulating the response to injury and atherosclerosis whereas rapid effects of estrogen that are mediated by ERs lead to the activation of endothelial nitric oxide synthase that result in vasodilatation [81]. Mice lacking either ERα or ERβ provide a powerful tool for the receptor studies in the heart and ERα and ERβ-null mice have been extensively studied for their cardiovascular phenotype. So far, the majority of animal studies have been focussed on vascular estrogen effects [81, 82]. The carotid artery injury model clearly demonstrated that the vascular injury was associated with a significant increase in vascular smooth muscle cell proliferation in both, wild- type and ERα- null mice. This increase was significantly attenuated by 17β- estradiol and no significant differences were observed between the genotypes. These results led to the conclusion that ERα is not required for mediating the inhibitory effects of 17β-estradiol in response to vascular injury and raised the possibility that ERβ might mediate the protective effects of estrogen [83].The carotid injury model was studied in ERβ- deficient mice as well involvement of ERβ in mediating the vasoprotective effects of estrogen has been examined. The degree of arterial injury was comparable between the both genotypes and could be attenuated by estrogen [84]. These results suggested that either of the two receptors ERα and ERβ is sufficient to achieve vascular protection. Alternatively, possibility of existence of the third estrogen receptor isoform “ERγ” should be taken into consideration.

Estrogen receptor expression in the heart and blood vessels is species-specific because both receptors, ERα and ERβ, could be detected human, rabbit and rat myocardium and aorta [85, 86]. Therefore, it is becoming increasingly clear that estrogens have direct effects on the myocardium that are independent of vascular hormone action. ER expression analyses showed species- specific tissue distribution pattern i.e., in rats, ERα was highly abundant in the myocardium and expressed at lower levels in the aorta whereas ERβ was highly expressed in both, aorta and myocardium [87]. Data about estrogen receptor expression in the mouse cardiovascular system remain controversial: the analyses by ribonuclease protection assay for ERα and ERβ mRNA in the mouse indicated only ERα transcripts in the aorta [88]. However, Iafrati *et al.* reported the detection of ERβ transcripts by RT-PCR in mouse aorta and blood vessels, including those of the αERKO [83]. The apparent contrast in ERβ detection between these two reports in the αERKO vasculature was most likely due to differences in the sensitivity of the techniques used, since ERβ mRNA was detected only by the more sensitive RT-PCR method. In contrast to ERα and ERβ messenger RNA, which could be detected in the mouse heart, immunohistochemical and protein expression analyses of estrogen receptors showed no detectable amounts of both receptors in the myocardium [89]. This data was also confirmed by experiments performed in our laboratory (personal observations and data provided by Arias- Loza, P.A). ERβ protein levels and lower ERα levels could be detected in endothelial cells in the mouse aorta [90]. Estrogen receptor exerts its function in the presence of its ligand which structure, function and biology are disscussed in the next section.

1.5. Natural and synthetic ER ligands

The estrogen receptor ligands fall into two groups: natural steroids and synthetic ligands. 17β- estradiol, a C_{18} steroid, is the most potent natural estrogen and is the major estrogen produced by the ovary. In the ovary, estradiol is produced by demethylation and aromatization of testosterone. Estradiol is also produced from estrone (E1), a less potent estrogen [91]. Estradiol and estrone are interconverted in many tissues. Estradiol is a non-selective estrogen receptor ligand which binds to both estrogen receptors with

similar affinity. Saturation ligand-binding analysis of human ERα and ERβ protein showed that both receptors bind radioactively labelled 17β- estradiol ([^{3}H]-17ß-estradiol (E_2)) with similar and high affinity [dissociation constant (K_d) = 0.05 - 0.1 nM] [92]. The other two endogenous estrogens- estrone (E1) and estriol (E3) also bind to estrogen receptors although with much lower affinity. Plant-derived, nonsteroidal compounds are termed phytoestrogens. Chemically, phytoestrogens can be divided into three major classes: flavonoids (such as genistein), coumestans (such as coumestrol); and lignans (such as enterodiol and enterolactone). Despite low estrogenic potencies, some phytoestrogens have been suggested to exert strong antiestrogenic effects, thereby inhibiting development of hormone-dependent cancers. Flavonoids as anti-cancerogenic agents could act via several alternative mechanisms, including induction of cancer cell differentiation, inhibition of protein tyrosine kinases, suppression of angiogenesis and direct antioxidant effects. These alternative mechanisms generally occur at flavonoid concentrations much higher (>5 µM) than the concentrations at which estrogenic effects are detected (<100 nM) [93].

Estrogen receptor ligands that act as ER agonists in some tissues but oppose estrogen action in the others are termed Selective Estrogen Receptor Modulators (SERMs). The SERM tamoxifen was initially considered to be a non- selective estrogen receptor antagonist because it competitively blocked estrogen binding to estrogen receptor and prevented its activation. Soon thereafter it was noted that agonistic/ antagonistic activities of tamoxifen were species- and tissue- dependent [94, 95]. During the seventies, the treatment of breast cancer with tamoxifen was fully established and tamoxifen became one of the basic substances used in adjuvant breast cancer therapy [96]. Several tamoxifen analogues such as toremifene, droloxifene, idoxifene and TAT-59 have been tested in various pre-clinical studies, but none of them has yet shown any significant advantage over tamoxifen in clinical trials for advanced breast cancer [4, 97]. Another SERM, raloxifen, is the only drug of this class that is currently approved for the treatment and the prevention of osteoporosis [98-100].

Synthetic compounds that can bind to either estrogen receptor isoform and competitively or noncompetitively activate or inhibit them are called estrogen receptor agonists or antagonists. The antagonists are also referred as Selective

Estrogen Receptor Down- Regulators (SERDs). The most potent SERD, ICI 182870 (Faslodex, Fulvestrant), is currently used as a pure non- selective ER antagonist in multiple *in vitro* and *in vivo* studies [101-103]. The Selective estrogen receptor down- regulators are distinguishable from SERMs, both pharmacologically and in terms of their molecular activity [104]. SERMs and SERDs are not estrogen receptor isoform specific, i.e. they can bind to both ERs with similar affinity.

ER isoform selective agonists and antagonists that bind specifically to ERα or ERβ represent another interesting and promising approach for dissecting the functional role of each ER isoform as an alternative to transgenic mouse models. Therefore, several ER- isoform specific agonists have been developed by B. Katzenellenbogen [105-107] and by Hillisch et al [108]. The ER isoform-selective agonists such as ERα agonist propyl pyrazole triol (PPT) [105], the ERβ selective agonists diarylpropionitrile (DPN) [109] and ERB-041 [110] are non-steroidal isotype- selective ER ligands. In contrast, the selective ER agonists 16α-LE_2 (ERα agonist) and 8β-VE_2 (ERβ agonist) developed by Hillisch and colleagues are steroidal ER agonists. These compounds were designed on the basis of the ER ligand binding domain structure and are structural derivatives of 17β- estradiol. A. Hillisch and colleagues extensively studied the structural differences between ERα and ERβ ligand binding pockets. The sequences of ERα and ERβ LBDs show 53% homology with only two amino acid deletions in the estrogen binding cavity of ERβ LBD: ERα L384 -> ERβ M336 and ERα M421 -> ERβ I373. Due to differences of the amino acid side chains (M volume of 85.9 $Å^3$ and L with the volume 82.6 $Å^3$ and I= 82.3 $Å^3$) it was predicted that the methionine side chain would allow to accommodate larger substituents. In the case of ERα agonist 16α-LE_2, a bulky lactone ring at the position 16α replaces the flexible M421 in ERαLBD (figure 3, section B) but cannot interact with I373 in ERβLBD. The ERβ agonist 8β-VE_2 contains a vinyl group in 8β position which fits well into ERβ ligand binding pocket, although colliding with L384 in ERα (figure 3, section C) [111].

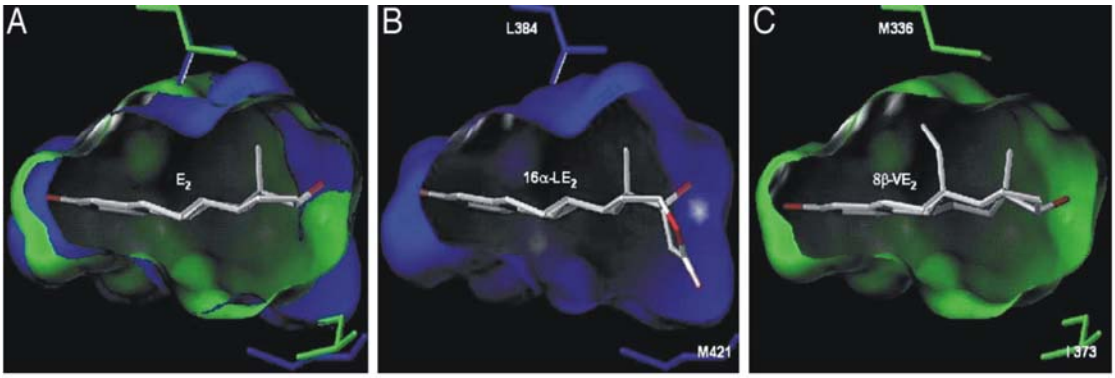


Fig. 3. Binding Modes of Steroidal Compounds within the Ligand Binding Pocket of ERα and ERβ. A, Superposition of the binding pockets of ERα (blue) and ERβ (green), including a side view of E2. The two amino acids differing in the binding pockets of ERα and ERβ are depicted. More space is available below the steroidal D-ring in the case of ERα and above the B- and C-rings in ERβ. B, 16α-LE2, the ERα agonist, fills the additional space below the D-ring (near amino acid M421) in the ERβ binding pocket with the lactone moiety. C, 8β-VE2, the ERβ agonist, fills the additional space above the steroidal B and C-rings (near amino acid M421) in the ERα binding pocket with the vinyl group. *From Hillisch, A et al. Mol Endocrinol 2004; 18: 1599-1609.*

Both agonists belong to the most potent and isotype- selective estrogens identified so far. *In vitro,* the ERα agonist 16α-LE_2 is characterized by 265- fold higher relative potency in ERα compared to ERβ- dependent transactivation assay. Receptor binding studies showed 70- fold preference of 16α-LE_2 for ERα. The agonist 8β-VE_2 is characterized by 190- fold higher relative potency on ERβ compared to ERα in ligand dependent transactivation assays. 8β-VE_2 showed up to 180- fold preference for ERβ comparing to ERα in receptor binding assays. The first *in vivo* tests of 16α-LE_2 and 8β-VE_2 were performed in rats and mice with particular attention to the reproductive system. It is known that ERα is predominantly expressed in uterus whereas ERβ is the dominant estrogen receptor isoform in ovaries. The isotype- selective ER ligands exhibited different effects: 16α-LE_2 but not 8β-VE_2 acted as a strong uterotrophic agent while 8β-VE_2 directly stimulated ovarian growth in rodents [108]. 16α-LE_2 and 8β-VE_2 have been characterized in *vitro* and *in vivo* and the results show that these ligands could be further investigated in the experimental disease models for their therapeutic potential. The effects of both substances on the cardiovascular system are presented in the current theses.

1.6. The role of aging in estrogen substitution efficacy and estrogen metabolism

Post- menopausal state is associated with decreased estrogen levels, elevated blood pressure and cardiac hypertrophy in women [112-114]. As mentioned before,

several studies suggested protective function of postmenopausal hormone replacement therapy (HRT) against heart disease with risk reductions up to 35-50% [115]. Nevertheless, controlled clinical trials such as HERS [6] and the recently terminated WHI trial [7] reported no protective role of conventional HRT for the primary or secondary prevention of coronary artery disease (CAD). In contrast to clinical trials, numerous experimental studies showed mostly protective effects of estrogen supplementation in animal models of human heart disease [116-118]. However these studies employed mostly young, ovariectomized animals. Therefore, the influence of aging on hormone substitution under experimental and clinical conditions remains poorly understood. Several rodent studies have employed aged animals and most of them, were focused on reproductive function [119], the central nervous system [116] and the bone phenotype[117]. Fortepiani et al analyzed ageing effects in spontaneous hypertensive rats [120]. Xu and colleagues studied left ventricular remodeling in 12- month old ovariectomized normotensive SD rats [121]. These studies were still focused on the phenotype of younger animals or very basic characteristics of senescent animals and the biological efficiency of estrogen substitution was not determined at that point. The only *in vitro* study implying senescent organs was described by Schriefers et al [122]. The authors analyzed the capability of young and senescent female rat ovarian tissue homogenates to generate sex hormones from precursor progesterone. Schriefers and colleques also analyzed the capacity of liver slices to generate sex- specific metabolites from testosterone. The results showed that ovarian homogenates could not produce measurable quantities of estrogen in both age groups. The generation of testosterone metabolites in liver slices displayed retarded activity in aged animals comparing to young ones.

Senescent spontaneously hypertensive rats represent the model of choice for hypertension associated cardiac hypertrophy in post- menopausal women. In contrast to the data obtained from young animal studies, estrogen metabolism in senescent animals is poorly studied and further studies are required to elucidate the mechanisms involved in the steroid hormone metabolism. Availability of new estrogen receptor isoform- selective agonists would help to reveal the role of either ER isoform in the senescent heart as well as their therapeutic potential.

1.7. Cardiac hypertrophy

Chronically elevated blood pressure (hypertension) is one of the major risk factors that induce cardiac hypertrophy. Cardiac hypertrophy is defined as abnormal cardiomyocyte growth and represents a physiological response that enables the heart to adapt to increased stress. However, the response can become pathological and ultimately lead to deterioration in cardiac function. Cardiac hypertrophy is also a physiological adaptation to training or pregnancy that enables the heart to enhance cardiac output. This type of hypertrophy is reversible and non- pathological. Chronic hypertension, however, causes pathological ventricular hypertrophy. Pathological hypertrophy is associated with alterations in the extracellular matrix. Increased mechanical stress together with neurohormonal stimuli lead to the activation of immediate early genes (c-fos, c-jun, c-myc) and fetal genes such as atrial natriuretic peptide (ANP), beta myosin heavy chain (βMHC), skeletal muscle actin and others. These genes are considered as typical hypertrophy markers [123]. Though under pathophysiological conditions both types of hypertrophic stimuli are present simultaneously, it has been shown that mechanical stretch alone is sufficient to induce the hypertrophic response in neonatal and adult cardiomyocytes [124]. Growth factors such as fibroblast growth factor (FGF), transforming growth factor beta (TGFβ), insulin growth factor (IGF) act through their membrane receptors and induce signal transduction events which lead to the normal postnatal cardiomyocyte growth and physiological hypertrophy. The members of the G- protein coupled receptor family, such as beta 1- adrenergic receptor (β1), the angiotensin II receptor (AT1) and the endothelin receptor (ET) also act as hypertrophic stimuli and have been related to the development of cardiac hypertrophy and heart failure.

1.8. Spontaneously hypertensive rats (SHR) as a model of cardiac hypertrophy

The spontaneously hypertensive rat (SHR) is a well established animal model of genetical hypertension leading to cardiac hypertrophy and heart failure. The SHR originate from Wistar Kyoto rats which were continuously selected for high blood pressure [125]. The rats develop high blood pressure reaching 171± 2 mmHg at the age of 7-15 weeks of age. At the age between 12 and 24 months the rats develop cardiac failure which includes reduced myocardial

performance and cardiac fibrosis [126, 127]. The SHR rats undergo “menopause” at the age of 12 months. In this model, no changes in SERCA2a protein expression was found, although altered calcium uptake was observed [128].

1.9. Myosin heavy chain

Myosin is the major contractile protein in the heart. It generates force by coupling its ATPase activity with cyclical interactions with actin. Myosin is a hexameric protein consisting of two myosin heavy chains (MHC) and two essential and two regulatory myosin light chains. There are two myosin heavy chain isoforms expressed in the rodent heart, i.e. alpha and beta- MHC (α- and β- MHC). Comparison of mammalian MHC α and β isoforms revealed 93% identity [129]. The expression of MHC isofoms is a dynamic process which is regulated during development, responds differentially to the hormones such as thyroid hormone, hemodynamic stress, exercise and also to cardiac hypertrophy [130-132]. Both isoforms possess different distribution patterns (atrial versus ventricular): αMHC is the major isoform in the atria and ventricles of healthy small animals. In contrast, βMHC is a major MHC isoform which is expressed in the fetal heart of rodents. Postnataly, βMHC is expressed in the hearts of large mammals and also in the hypertrophied hearts of rodents [133]. MHC generates contraction by coupling its ATPase activity with cyclic interactions with actin. Myosin heavy chain isoforms differ in their mechanical and enzymatic properties: αMHC possesses higher ATPase activity comparing to βMHC. In an *in vitro* motility assay, individual actin filaments in contact with αMHC move two to three fold faster than those in contact with βMHC regardless of the mammalian species. In contrast to contraction velocity, average force generated by both MHC isoforms is species specific: in rabbits, despite higher velocity of αMHC, it generates approximately the half of maximal force compared to that of βMHC. In rats, both MHC isoforms generate similar maximal force [134]. In rats, both MHC isoforms generate similar maximal force [134]. Myosin heavy chain (MHC) isoform expression is species specific, i.e. small rodents, such as adult mice and rats predominantly express αMHC whereas βMHC is the dominant isoform in bigger mammals (rabbits, pigs and humans). Therefore, the ratio between αMHC and βMHC isoforms plays a major role in the determination of cardiac contraction velocity and force generation.

Additionally, the shift of α- to- β- MHC ratio towards βMHC in the rodent heart serves as a molecular marker of cardiac hypertrophy.

2. Aims of the study

This work was designed to address the following questions:

1. What role plays each estrogen receptor isoform (ERα and ERβ) in cardiac hypertrophy? Which mechanisms are involved in genetical and/ or physiological responses?

Spontaneously hypertensive female rats were used an *in vivo* model of cardiac hypertrophy. Selective activation of each ER isoform was studied using selective ERα (16α-LE_2) and ERβ (8β-VE_2) agonists.

2. Does ageing influence estrogen substitution efficacy? If yes, then which mechanisms play a role in altered estrogen substitution efficacy?

The 22-24 months old SHR female rats served as a model of post- menopause associated cardiac hypertrophy. The substitution with 17β- estradiol as well as selective estrogen receptor activation was carried out and the results were compared to the data obtained from the study with young female SHR. The latter served as “baseline” information for the comparative analysis. Extensive analysis of estrogen metabolism in young and senescent SHR rats together with identification of proteins which could be involved in estrogen metabolism helped to elucidate several mechanisms which could be involved in estrogen metabolism.

3. Which proteins could directly interact with estrogen receptor alpha in the heart and how does this interaction influence the function of both proteins?

One of the non- genomic estrogen receptor action mechanisms comprises its interaction with other proteins. Therefore, the identification of still unknown ERα interaction partners was performed using bacterial two- hybrid system. The neonatal human cDNA library was screened using ERα ligand binding domain which contains protein- protein interaction motif. The co-localization of both proteins, their physical interaction verification together with functional analysis are presented in the theses.

3. Materials and Methods

3.1. Study design

For the in-vivo studies, 17β- estradiol (E2), selective ERα agonist 3,17-dihydroxy-19-nor-17α-pregna-1,3,5 (10)-triene-21,16α-lactone (16α-LE_2) and the selective ERβ agonist 8-vinylestra-1,3,5(10)- triene-3,17β-diol (8β-VE_2) were used. 17β-estradiol was obtained from Sigma and the agonists were provided by Schering AG (Berlin). The structural formulas of all three substances are shown in Figure 4.

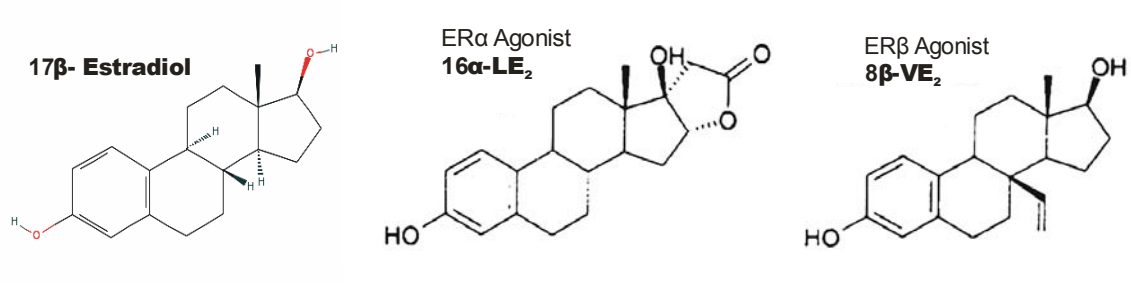


Figure 4. Structure of 17β- estradiol and selective estrogen receptor agonists.

3.2. Animal model and treatment

For animal experiments, the female spontaneous hypertensive (SHR/Ncr1Ico) rats were used. The animals were obtained form Charles River Laboratories in France. At the beginning of treatment, the young rats were 12-14 weeks old and senescent rats were 22-24 months old. Prior to treatment, the rats were kept in the experimentation room (Days -7 to -1). The animals were randomly selected and repartitioned in the study groups. All animals (prior and during the study) were kept under standard conditions: 12 hours on/off light cycle, commercial diet and water *ad libitum*. In order to eliminate the effects, caused by endogenous estrogen, rats were ovariectomized or sham operated prior to treatment under isoflurane anesthesia. The study consisted of five groups: sham operated animals (sham), ovariectomized (ovx) and placebo treated, ovariectomized and substituted with 17β-estradiol (ovx+E2), ovariectomized and treated with the ERα agonist 16α-LE_2 (ovx+ αAg) and ovariectomized treated with ERβ agonist 8β-VE_2 (ovx+ βAg). The treatment scheme and surgical operations performed on the rats were following: sham group+ placebo (95 µl peanut oil+ 5 µl ethanol),

ovariectomy (ovx) group+ placebo, ovx+ 17β- estradiol (2 µg/kg of body weight/day), ovx+ 16α-LE_2 (30 µg/kg of body weight/day), ovx+ 8β-VE_2 (30 µg/kg/day). All the substances were dissolved in 100% ethanol. Prior to subcutaneous injections, the stock solutions were dissolved in peanut oil. The rats were treated for 12 weeks on daily basis. All surgical interventions, treatments and subsequent invasive hemodynamic analysis were performed in accordance with laboratory animal treatment directives and with the agreement of local laboratory animal protection committee.

3.3. Morphometric analysis

After 12 weeks of treatment, the determination of body weight, heart weight and uterus weight and the tibia length were performed. Cardiac hypertrophy in all animals was determined by measuring heart weight and normalizing it to tibia length.

3.4. Hemodynamic analysis

The hemodynamic measurements were performed according to published protocols after 12 weeks of treatment under light isoflurane anesthesia and spontaneous respiration under isoflurane anesthesia using 1.5vol % isoflurane supplemented with 0.5 l oxygen per minute. Pressure curves were measured using fluid-filled PE50 tubing connected to a microtip manometer (Millar Instruments) inserted via the right carotid artery and calibrated to mid-chest level. Left ventricular pressure curves were recorded after catheter placement in the left ventricular (LV) cavity, systolic and diastolic blood pressure measurements were obtained upon catheter withdrawal in the thoracic aorta. During positive pressure ventilation following a midsternal thoracotomy, a calibrated flowmeter (2.5 mm; Statham) was placed around the ascending aorta for continuous measurement of aortic blood flow (cardiac output). Measurements were performed by a trained observer blinded for treatment groups. The following parameters were obtained during hemodynamic measurements and evaluated: left ventricle systolic pressure, left ventricle end- diastolic pressure, heart rate, cardiac output, systolic aortic pressure, diastolic aortic pressure and mean of aortic pressure.

3.5. Determination of serum hormone levels

After hemodynamic measurements, the blood samples of each rat were collected in citrate tubes for blood clotting analysis, centrifuged at 800xg at room temperature for 10 minutes and the upper (plasma) phase was collected and stored at -80°C until usage. A day before hormone measurements, the frozen samples were defrozen overnight at +4°C. The estrogen (E2), estrone (E1), total testosterone (T total), free testosterone (T free), dihydrotestosterone (DHT), dehydropepiandrosterone (DHEA) and androstendione (ASD) plasma levels were determined using double antibody radioimmunoassay kits provided by Diagnostic Systems Laboratories, Inc according to the manufacturer's protocol. Cross- reactivity of 3rd generation estradiol RIA kit was 6.9% with estrone, other cross- reactivity was not detectable. Estrone RIA cross- reacted with E2 (1.25%) and did not cross- reacted with other hormones. Cross- reactivity of total testosterone RIA with dihydrotestosterone was 6.6%, with androstenedione 0.9% and not detectable with E2. Free testosterone assay did not show substantial cross- reactivity with the hormones measured in this project. The dihydrotestosterone RIA cross- reacted with testosterone (0.6%) and did not cross- react with other hormones. The DHEA RIA cross- reacted with androstenedione (0.46%) and testosterone (0.028%). Androstenedione RIA kit cross- reacted with the following hormones: dihydrotestosterone (0.08%), DHEA (0.07%) and estrone (0.03%). The measurements were performed according to the manufacturer's protocol. The results are presented as mean (pg/ml)± SEM.

3.6. Molecular analysis

3.6.1. Myosin heavy chain electrophoresis

Native proteins samples for alpha and beta myosin heavy chain (α/β MHC) protein expression analysis were electrophoretically separated and subsequently visualized by silver staining. The protein samples were prepared according to the following protocol: rat hearts were rinsed in ice- cold PBS, 20mg of rat cardiac tissue was homogenized in 200 µl ice- cold sample buffer containing a protease inhibitor cocktail (Roche). Homogenates were centrifuged at 12000x g, +4°C, 30 min. The supernatant was stored at -80°C. Protein concentration was determined using Bradford assay reagent (Bio Rad). Standard curves were generated using

known concentrations of BSA. 500 ng of each protein sample was fractionated on 6% polyacrylamide SDS separating gel, containing 5% glycerol. The electrophoresis was run at 130 V for 18 hours at +4°C. After electrophoretic protein separation, the gels were subjected to silver staining using Silver Stain Plus Kit (Bio Rad). After staining, α- and β- MHC appeared as two separate bands which were scanned and densitometric analyses of bands were performed using the ScanPack3.0 software. The statistical evaluation of the data as performed and the data is presented as the ratio α/β MHC± SEM.

3.6.2. Generation of rabbit polyclonal antibodies against estrogen receptor beta

Rabbit polyclonal ERβ antibodies were generated to assess ERβ protein expression. Immunogen peptide comprising amino acids 394- 409 of the R. norvegicus estrogen receptor beta (NCBI acc. nr. NP_036886) was synthesized to which additional cystein residues were added and the peptide C-WVIAKSGISSQQQSVR was coupled to the carrier. Prior to immunization, pre-immune serum was collected. Primary immunization of the rabbits with purified peptide (16 aa, 70-80% purity), boosting and antiserum collection were performed. The specificity of the antiserum for ERβ was tested using peptide blocking ("antibody competition") assay. A reaction mixture contained 1µl/ml of antiserum and either 5 µg of specific (ERβ) or unspecific (ERα) peptides. The reaction mixtures containing either ERβ antiserum+ ERα peptide, antiserum+ ERβ peptide or antiserum only were incubated overnight at 4°C in PBS. Antibody- antigen complexes were pelleted by centrifugation at 12000xg for 15 min. (4°C). The supernatant was carefully removed and resulting pellets were dissolved in 1 ml of PBS-Tween 20 containing 5% non- fat milk and used for Western blotting. The bands were visualized using secondary HRP- conjugated donkey anti- rabbit antibody (Amersham) and using ECL™ Western Blotting Detection Reagent Kit (Amersham Biosciences). Equal protein loading was verified by GAPDH expression using mouse monoclonal anti- GAPDH antibody (Chemicon) and the bands were visualized by sheep anti- mouse antibody conjugated with horse reddish peroxidase and using ECL™ kit for signal development. Antiserum specificity and antibody affinity were tested in rat heart protein samples (40µg /lane) that were electrophoretically separated in 8% PAGE

SDS gel and subsequently transferred onto nitrocellulose membrane. ERβ antiserum without peptide as well as antiserum preincubated with ERα peptide, recognized rat ERβ while antiserum pre-treated with specific peptides failed to detect ERβ (Fig. 5).

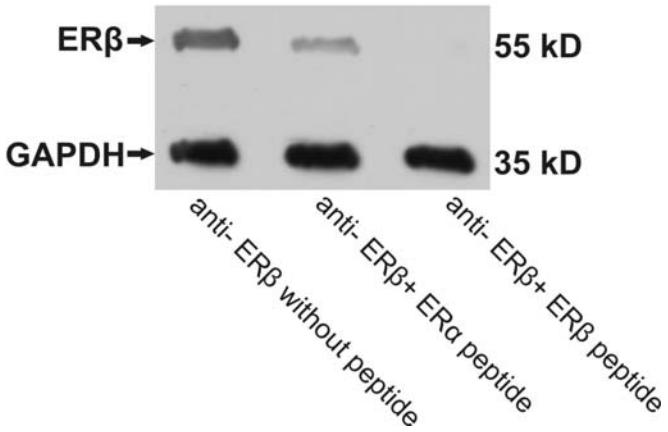


Figure 5. Antibody competition assay of rabbit polyclonal ERβ antiserum (antibody competition assay). ERβ antiserum was specifically blocked by ERβ peptide while antiserum alone and/ or incubated with ERα peptide could efficiently recognise its antigen in cardiac protein lysate. ERα peptide showed slight cross-reaction with antiserum possibly due to substantial excess of antigen over antibody.

3.6.3. Protein expression analysis by Western blotting

Depending on the target protein molecular weight, proteins were separated on 7%-12% polyacrylamide SDS gels with subsequent electrophoretical transfer to the nitrocellulose membranes. After transfer, membranes were dried on Whatmann filter paper to fix the proteins on the membranes. The non- specific background was blocked at least 1hour at room temperature with 5% non- fat milk power in PBS/Tween 20. The primary antibodies were diluted in the background blocking solution and membranes were incubated with antibody solution at least one hour at room temperature or, if necessary, overnight at +4°C with gentle agitation. Afterwards, membranes were washed 4-5 times with large volume of PBS/T with subsequent incubation with diluted HRP-conjugated secondary antibody for 1 hour at room temperature. Unbound antibodies were washed with large volumes of PBS/T. Proteins were detected using enhanced chemiluminescence reagent (ECL) according to the manufacturer's protocol. The following antibodies were used in protein expression analysis: anti- estrogen receptor alpha (rabbit polyclonal, ER-21, a generous gift of prof. G. Greene, University of Chicago), rabbit polyclonal anti- ERβ generated in our laboratory

(ER Beta-3919, #6040), anti-ERα (MC-20, Santa Cruz), anti-GAPDH (Abcam), anti- αB crystallin (StressGen), anti- phospholamban (ABR), anti- phospho-phospholamban (Upstate Biotechnology), anti- SERCA 2 (Abcam).

3.6.4. Immunofluorescent staining

The mouse liver tissue samples were fresh frozen in liquid nitrogen and cut with a cryostat. The 5 µm thick sections were kept frozen at -20°C until fixation. The sections were fixated 4% paraformaldehyde (PFA) solution for 30 min at 4°C and permeablized with 0.1% Triton X-100 solution for 10 min. at room temperature. After permeabilization, the sections were rinsed several times with PBS and blocked with normal 5% goat serum (diluted with PBS) 30 min. at room temperature. ERα expression in mouse liver sections was detected using rabbit polyclonal anti- ERα (ER21) which was diluted in 5% goat serum to final concentration of 2 µg/ ml. The sections were incubated with the primary antibody overnight at room temperature in the humidity box. The sections which were used as negative control were incubated in diluted normal goat serum. Next day, all sections were washed with PBS and incubated for 30 min. with goat anti-rabbit AlexaFluor 594 secondary antibody (Molecular Probes, Invitrogen). The final concentration of the secondary antibody was 1.5 µg/ml. The sections which were incubated only with secondary antibody served as a negative control. After incubation with the secondary antibody, all sections were extensively washed with PBS and mounted using Vectashield® mounting medium (Vector Labs). The sections were examined using fluorescence microscope.

17β- HSD10 and COXI co- localization in neonatal rat cardiomyocytes was identified using immunocytochemical method. Prior to the immunostaning, neonatal cardiac myocytes were cultivated for 2-3 days in Lab-Tek™ Chamber Slides™ (Nunc). Before fixation, cell growth medium was discarded, the cells were washed several times with PBS and fixated with 4% PFA at 4°C for 30 min. Subsequent steps, such as permeabilization, background blocking and incubations with the antibodies, are described in the first paragraph of this section. The following antibodies were used for co- localization studies: rabbit polyclonal anti- 17β- HSD10 (generous gift of Yang S.-Y, New York State Institute for Basic Research in Developmental Disabilities, New York, USA.) and mouse monoclonal anti- COXI (Molecular Probes). BLCAP10 and ERβ in

neonatal cardiac myocytes were detected using rabbit polyclonal anti- BLCAP10 (generous gift of I. Gromova, Institute of Cancer Biology, Danish Cancer Society, Copenhagen, Denmark) and mouse monoclonal anti- ERβ (generous gift of G. Greene, University of Chicago). Goat anti- mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488 were purchased from Molecular Probes (Invitrogen). The microscopy analyses were performed using confocal microscope.

3.7. Construction of a bait vector containing rat estrogen receptor alpha ligand binding domain (rERα_LBD)

3.7.1. Preparation of total rat heart RNA

Total rat heart RNA was prepared using Trizol reagent according to the manufacturer's protocol. Rat heart was briefly rinsed with ice- cold PBS. 100 mg of myocardium was frozen in 1 ml of Trizol at -80°C until usage. Thawed heart sample was homogenized and 200 µl of chlorophorm was added. After vortexing, the probe was centrifuged at 12000xg for 15 minutes at +4°C and the upper transparent phase, containing RNA, was carefully discarded. RNA was precipitated with isopropanol and centrifuged for 10 min. at 12000xg, +4°C. The RNA containing pellet was washed with 70% ethanol and centrifuged for 5 minutes at 7500xg, +4°C. The resulting RNA pellet was dissolved in sterile water, treated with DEPC. RNA concentration was estimated, aliquoted and stored at -80°C until usage. The RNA quality was estimated by electrophoresis in 1% agarose gel, containing formaldehyde. The electrophoresis was run in MOPS buffer at 80 V. The integrity of 18 S and 28 S rRNA bands served as RNA quality marker.

3.7.2. Amplification of rat ERαLBD

R.norvegicus estrogen receptor alpha mRNA (NCBI accession number X61098.1) was used as reference sequence for primer design. The following primers were designed for amplification of ERαLBD: forward primer 5'- GCA-TCG-AAT-TCC-TTA-TTG-ACC-AAC-CTG-GCA-GAC-3' (contains 5'-terminal EcoRI restriction site) and reverse primer 5'- CGA-GGA-TCC-TTC-TCA-GAT-GGT-GTT-GGG- 3', containing 3'- terminal BamHI restriction site. One- step RT-PCR kit (Qiagen) was used for this PCR. The PCR reaction mixture contained 1

µg of template RNA, 0.6 µM of each primer, 1x One- step PCR reaction buffer, 400 µM of each dNTP, 1x Q- solution, water and One- step RT-PCR Enzyme mix. The total volume of one reaction mixture was 50 µl. The PCR reaction was performed under following conditions:

Reverse transcription:	50°C	30’
Initial PCR activation step:	95°C	15’
3 step cycling (30 cycles):		
	95°C	30’’
	52°C	30’’
	72°C	1’
Final extention:	72°C	10’.

The PCR products were analyzed on 1% agarose gel. The electrophoresis was run in 1x TAE buffer at 70V. The expected PCR product was 780 bp. It corresponds to bp 1121- 1879 of LBD and 5’- terminus of rat estrogen receptor alpha mRNA with EcoRI and BamHI restriction sites at 5’ and 3’ ends respectively. Following electrophoresis, the PCR product was isolated from gel using DNA gel extraction kit (Qiagen) according to the manufacturer’s instruction. The cleaned PCR product was subsequently subcloned into a pCR II cloning vector.

3.7.3. Ligation and transformation of One- shot competent bacterial cells

The PCR product was ligated into PCRII vector (Invitrogen) using T4 ligase (Fermentas). The overnight ligation was performed at +14°C. After ligation, 1 µl of ligation reaction was incubated with 50 µl of INVαF’ One Shot competent cells on ice. The bacterial competent cells were heat shocked at 42°C for 45 sec, 1 ml of LB medium was added and cells were growing at 37°C for at least 1 hour in a shaking incubator. Aliquots of transformed bacteria were grown overnight at 37°C on LB- agar plates containing ampicillin. The pCRII_ERαLBD vector harbours ampicillin resistance gene and X-Gal and IPTG for blue- white selection. The white colonies were selected for further analysis. Plasmid DNA was extracted and restriction analysis was used to detect the vectors containing ERα_LBD insert.

3.7.4. Construction of bait vector containing ERα_LBD insert

The bait vector was generated using BacterioMatch™ Two- Hybrid vector system (Stratagene). Briefly, pCRII recombinant vector containing ERαLBD insert was digested with EcoRI and BamHI and the ERαLBD insert was ligated with the digested bait vector, pBT. DH5α cells were transformed with 1 µl ligation reaction product and transformants were grown on LB- agar, containing chloramphenicol. The colonies grown on the plates were selected for further analysis. Plasmid DNA was extracted and subjected to restriction analysis, colony PCR and sequencing. The bait vector, containing ERαLBD was selected for further screening of human neonatal heart cDNA library. The pCRII and pBT vector maps are shown in the figure 6, panels A and C, respectively. The representative agarose gel showing digested pCRII containing ERαLBD insert and linearized pBT plasmid are represented in panels B and D.

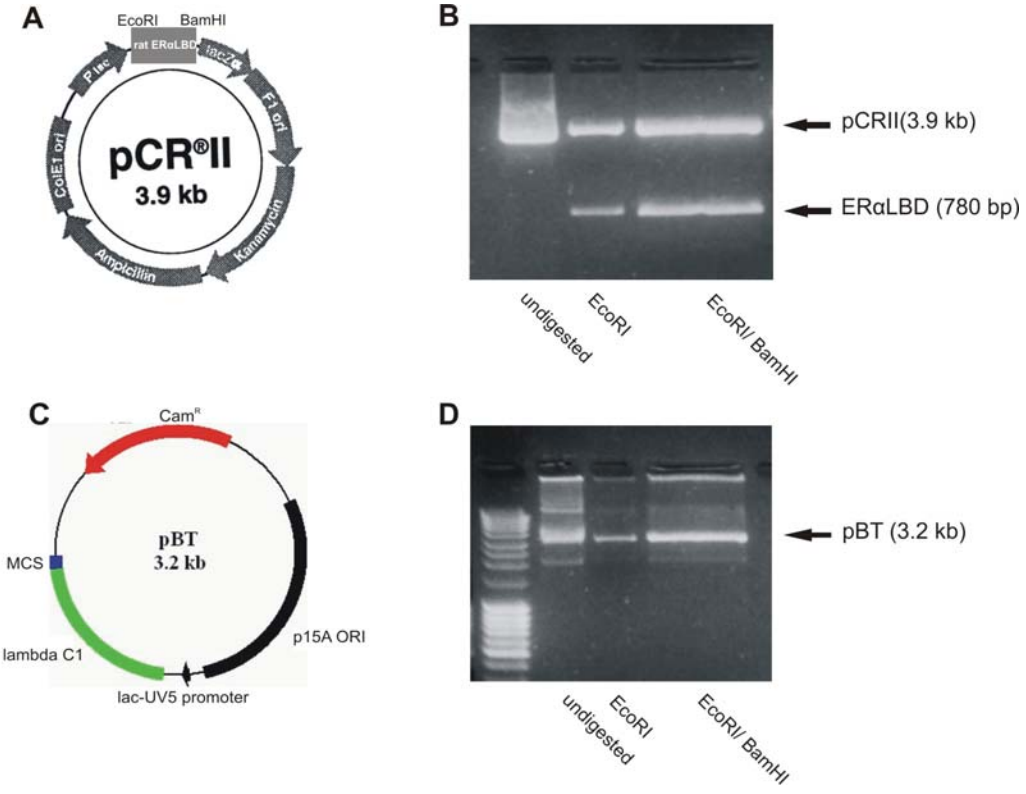


Figure 6. The pCRII vector containing ERαLBD insert and bait "backbone" vector (pBT). The ERαLBD fragment was cut out of pCRII plasmid (A) with EcoRI and BamHI restriction enzymes. The restriction product was electrophoretically separated on agarose gel (B). The bait vector was linearized with BamHI and EcoRI (C) and run on agarose gel (D).The ERαLBD was ligated into pBT vector using T4 ligase.

Prior to the neonatal heart cDNA library screening, the recombinants containing the DNA insert in frame with the λcI, were identified and the pilot protein

expression analysis in the BacterioMatch® Two-Hybrid System reporter strain (RS) Competent Cells (Stratagene) was performed. The transformed bacteria were cultivated in S.O.C. medium for 1 hour at 30°C and plated on LB-chloramphenicol agar plates. The plates were incubated for 17-30 hours at 30°C. Fresh LB broth containing chloramphenicol was inoculated with individual colonies grown on LB/ chloramphenicol plates and protein expression was induced by adding 5 µM IPTG and grown at 30°C until OD_{600} was 0.5- 0.6. Transformed Reporter Strain cells that were not treated with IPTG served as a negative control. 20 µl of the cell suspension was mixed with the same amount of Laemmli protein sample buffer (Bio- Rad) containing 5% beta-mercaptoethanol (βME), boiled for 5 minutes at 95°C and electrophoretically separated in 10% SDS-PAGE gel. The protein expression in reporter strain competent cells was verified by Western blotting using anti- λC1 antibody (Invitrogen) as shown in the figure 7.

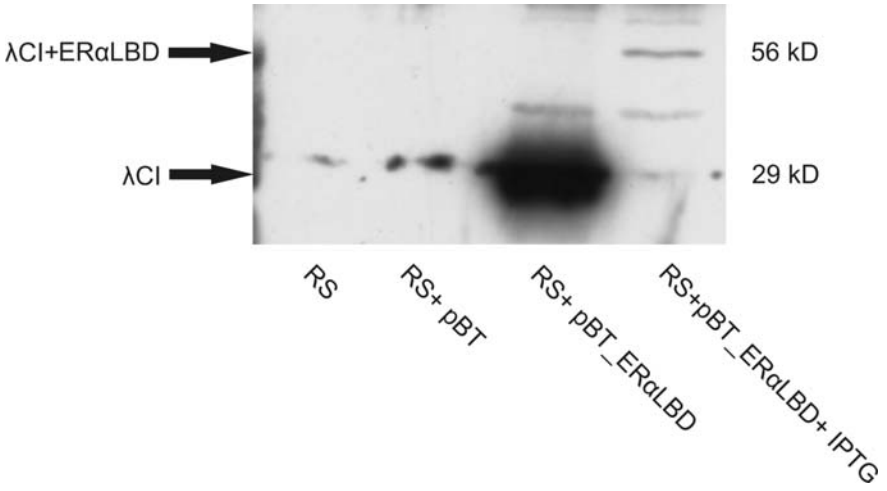


Figure 7. ERαLBD protein overexpression in Reporter Strain competent cells. Reporter strain (RS) bacteria were transformed with the pBT vector containing estrogen receptor alpha ligand binding domain. Non- transformed RS cells as well as pBT vector without insert served as a negative control. Basal λCI expression was observed in RS containing recombinant bait vector (lane 3), whereas fusion protein containing λCI *and* ERαLBD (56kD) could be detected in RS treated with IPTG. λCI (29kD) was detected using anti- λCI antibody.

3.8. ERα ligand binding domain protein- protein interaction partners in the heart

3.8.1. Neonatal human heart cDNA library screening for ERα protein-protein interaction partners

After recombinant bait vector and cDNA library in target vector were available, further cDNA library screening was performed using Reporter Strain (RS) Competent cells. The particular feature of the strain is the presence of the reporter cassette, consisting of the lacZ and ampicillin resistance gene tandem, in its genome. The recombinant pBT expresses the lambda CI (λCI) tagged to the "bait" protein. The design and generation of recombinant bait vector containing rat ERαLBD are described in detail in *Materials and Methods,* section 3.4*.* The recombinant target vector (pTRG) contains RNA polymerase α subunit (RNAP alpha) gene tagged to the unknown cDNAs from the cDNA library. The reporter cassette in RS can be expressed only in the case of interaction of ERαLBD and unknown protein coded in the cDNA library. The target vector confers tetracycline resistance and directs the transcription of N- terminal domain of RNA polymerase α subunit. The bait vector encodes full-length bacteriophage λ repressor protein (λcI), containing N- terminal DNA- binding domain and C-terminal dimerization domain. In the case of bait and target interaction, they recruit and stabilize the binding of the RNA polymerase at the promoter and activate transcription of a reporter gene cassette which consists of ampicillin resistance gene and β- galactosidase gene. Protein- protein interaction affinity (strength) between bait and target proteins strongly correlates with the gene expression levels of reporter gene. The schematic overview of BacterioMatch II two- hybrid system *modus operandi* is shown in Figure 8.

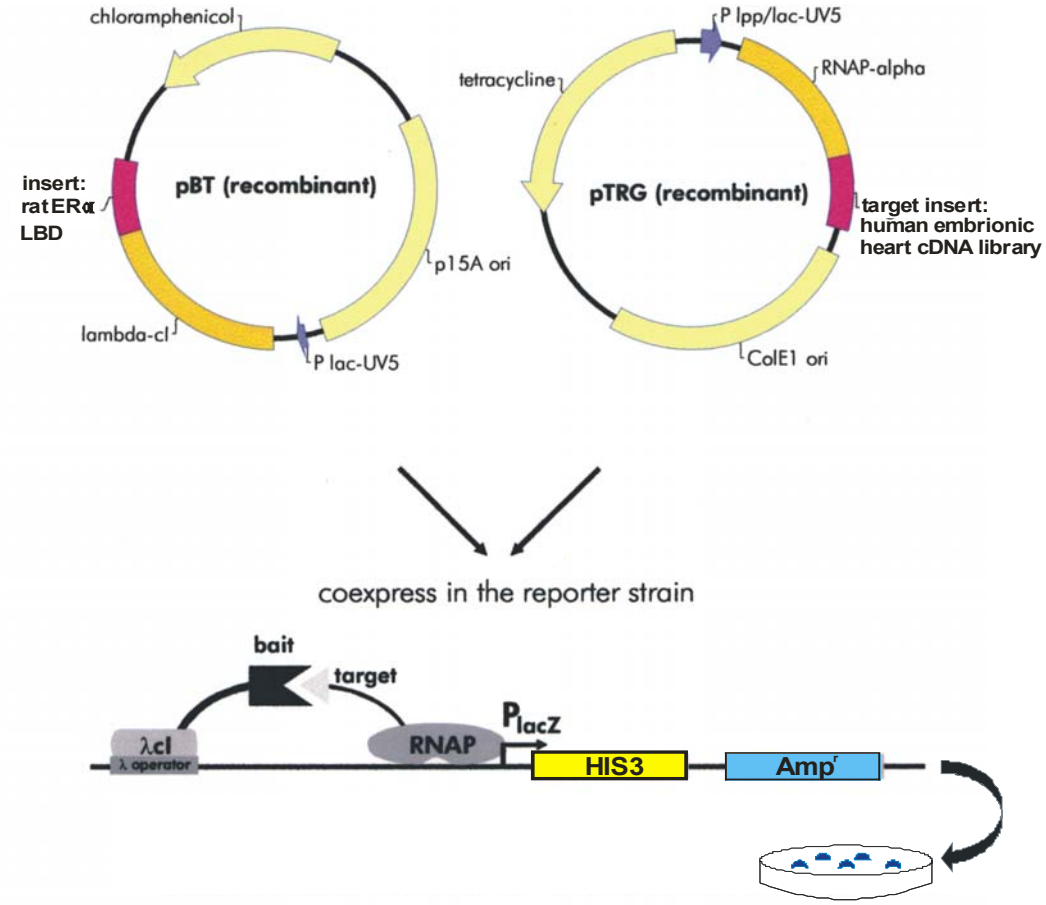


Figure 8. The scheme of BacterioMatch –Two hybrid system. Both, recombinant bait vector (pBT) containing rat ERα LBD and the pool of target vectors (pTRG) containing embryonic human heart cDNA library, are co-transformed into Reporter Strain (RS) competent cells. The protein- protein interaction initiated the expression of reporter cassette containing lacZ gene and ampicillin resistance gene. The resulting positive clones grown on LB-agar containing carbenicillin and are blue.

The human neonatal heart cDNA library compatible with a bacterial two hybrid system was generated from human neonatal heart cDNA pool which was cloned unidirectionally into the pTRG XR- target vector. Reporter strain competent cells were co-transformed with cDNA library plasmids and pBT vector containing ERαLBD and the dark- blue colonies were analyzed. They were grown in LB medium containing appropriate antibiotics, plasmid DNA was extracted and sequenced using pTRG forward primer (5'-TCC GTT GTG GGG AAA GTT ATC-3'). Sequence similarity search was performed using Basic Local Alignment Search Tool (BLAST) in the homepage of National Centre of Biotechnology Information (http://www.ncbi.nlm.nih.gov).

3.8.2. Cloning of ERαLBD protein- protein interaction partners candidate genes

The following candidate proteins were selected for further analysis:

1. 17β hydroxysteroid dehydrogenase type 10 (17βHSD10);

2. Bladder cancer associated protein 10 (BLCAP10);

For further cloning procedures, the Gateway™ cloning technology was used. The primers contained attB recombination sites and gene- specific sequences. The following primer pairs were designed:

1. 17β- hydroxysteroid dehydrogenase type 10 HSD10:

Forward primer: 5'- GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT GAT GGC AGC AGC GTG TCG GAG CGT G-3';

Reverse primer: 5'- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TCA AGG CTG CAT ACG AAT GGC CCC ATC CAG-3';

2. Bladder cancer associated protein 10 (BLCAP10):

Forward primer: 5'- GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GTA TTG CCT CCA GTG GCT GCT G- 3';

Reverse primer: 5'- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA GGT GCC CAC AAC GCC GGG- 3'.

The amplification of 17βHSD10 and BLCAP10 cDNA was performed using Advantage 2 PCR system (BD Biosciences). The PCR reactions were run under following conditions:

denaturing step:	95°C, 1'
two- step cycle:	95°C, 30''.
	68°C, 2'
	30 cycles
final extension:	62°C, 1'.

The PCR product was electrophoretically separated and extracted. The DNA concentration was determined and 100 ng of gel- extracted PCR products were used for the construction of recombinant entry vectors containing human 17βHSD10 and BLCAP10, respectively.

3.8.3. Generation of recombinant human 17β- hydroxysteroid dehydrogenase type 10 protein

The expression vectors were generated using the Gateway™ system developed by Invitrogen. Target cDNA was subjected to the recombination reaction with the donor vector (pDONR) and the reaction mixture was incubated 1 hour at 25°C following enzyme inactivation by Proteinase K. DH5α competent cells were transformed with the recombinant vector and grown on LB agar plates containing kanamycin. Resulting colonies were analyzed using colony PCR. The colonies, containing insert were further grown in LB medium, containing kanamycin and plasmid DNA was extracted. Extracted DNA was subjected to further restriction analysis using NruI and HpaI restriction enzymes. The entry vector containing 17βHSD10 cDNA insert was used for further recombination reactions with the protein expression vectors. The protein expression vectors for mammalian cells as well as E. coli contained either N-terminal GST (pDEST™ 27 for mammalian cells and pDEST™ 15 for E.coli), N-terminal- 6x His tag (pDEST™ 26 for mammalian cells and pDEST™ 17 for E.coli), or no tag (pDEST™ 14 for E. coli). The recombination reaction products were inserted into Library Efficiency® DH5α™ cells (Invitrogen) by heat- shock and the transformants were grown overnight at 37°C in S.O.C. medium containing ampicillin. Resulting colonies were screened for the positives ones. The pDEST14_, 15_ and 17_17βHSD10 plasmids for protein expression in E.coli system were sequenced using T7 primer. The recombinant vectors were inserted into BL21 E. coli strain for protein overexpression. The cells were grown in LB medium containing 100ml/ ml ampicillin until they reached mid- log phase (OD 0.4), the aliquots were taken for further analysis. Protein expression was induced with 0.2% arabinose for 2, 3 and 4 hours. Bacterial aliquots were dissolved in Laemmli buffer electrophoretically separated in 10% PAAG gel. The protein bands were visualized by staining with coomassie brilliant dye. 17βHSD10 cDNA was transferred into destination vectors (pDEST) containing CMV promoter. The resulting recombinant pDEST vectors contained cDNA insert with either N-terminal His or N- terminal GST affinity tag. The constructs were propagated in DH5α bacteria. The extracted plasmid DNA was subjected to primary restriction analysis and sequenced using following primer: TGGCGTACTACCATCACCAT. The plasmids containing 17βHSD10 cDNA insert were amplified in DH5α cells with subsequent plasmid DNA extraction. Human cervix carcinoma (HELA) cells

were transfected with the 17βHSD10 protein expression vector using LipofectAmine PLUS™ system (Invitrogen). 24 hours after transfection, cells were harvested in RIPA buffer containing Protease Inhibitor cocktail (Roche), denatured in protein sample and subjected to protein electrophoresis. The overexpression of 17β-HSD10 protein containing either GST or His tag was verified using anti- GST (Santa Cruz) or anti- His (Upstate Biotechnologies) antibodies.

3.8.4. Generation of recombinant vectors for mammalian expression of rat ERα_LBD

The recombinant vectors, containing rat estrogen receptor ligand binding domain (rERαLBD) were generated using a Mammalian Expression System (Invitrogen). The bait vector containing rat rERαLBD and used in BacterioMatch™ Two Hybrid System (Stratagene) experiments (section 3.7.4.) served as a template for generation of rERαLBD containing attB recombination sites. The following primers for PCR reaction have been generated:

attB1_ERαLBD_F (forward primer):

5'- GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGG CTT ATT GAC CAA CCT GGC AGA C- 3';

attB2_ ERαLBD_R (reverse primer):

5'- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTC TCA GAT GGT GTT GGG GAA GCC-3'.

Underlined sequences are estrogen receptor ligand binding domain- specific whereas non- underlined parts of the primers contain recombination sites. PCR reaction mixture was prepared using BD Advantage™ 2 PCR Enzyme System and the DNA fragment was amplified under following conditions:

1. Initial denaturation: 95°C, 1 min.

2. Denaturation: 95°C, 30 sec.

3. Annealing: 50°C, 1 min.

4. Elongation: 68°C, 1 min.

Steps 2-4 were repeated 30 times.

5. Final extension: 68°C, 1 min.

The PCR product was electrophoretically separated in 1% agarose gel and purified. The entry vector containing rat ERαLBD (BP reaction) was generated and further inserted into expression vectors. The following recombinant vectors were generated: pDEST12.2_rERαLBD (for native protein expression), pDEST26_rERαLBD (N-terminal Histidine Fusion protein) and pDEST27_rERαLBD (N-terminal GST fusion protein). The protein overexpression was tested in HELA cells that were transfected with all 3 vectors described above. The transfected cells were harvested in Laemmli buffer, and subjected to electrophoretical separation in 10% SDS- polyacrylamide gel. Thereafter, the recombinant rat ERαLBD was visualized using rabbit polyclonal anti- ERα (MC-20, Santa Cruz) antibody which recognition site is located in ligand binding domain of ERα (data not shown).

3.8.5. Mitochondria preparation for 17βHSD10 enzymatic assay and Western blot analysis

Enriched mitochondrial fraction from mouse and rat heart was prepared according to the modified protocol developed by RD Systems. Rat hearts were briefly washed in ice- cold isotonic solution and then homogenized in 5 ml of Buffer A (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 1 mg/ml of fatty acid- free BSA, 10 mM HEPES- NaOH pH 7.4). The resulting homogenate was diluted with 5 ml of Buffer A and centrifuged for 10 minutes at 600xg (4°C). The liquid phase was transferred to a new tube and centrifuged at 14000x g for 30 min. at 4°C. The supernatant was discarded and the pellet containing mitochondria was resuspended by pipetting in Buffer B (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 10 mM HEPES- NaOH, pH 7.4) and then centrifuged for 15 min. at 15000xg (4°C). The mitochondrial pellet was resuspended in 2 ml of RIPA buffer containing protease inhibitor cocktail (Roche) and frozen at -20°C until usage.

3.8.6. 17βHSD10 enzymatic assay

Measurements of recombinant 17βHSD10 enzymatic activity were described by Yang S.-Y. et al [135, 136]. The protocol was modified for endogenous 17βHSD10

oxidase activity measurements. The reaction mixture contained assay buffer (0.1 M K_2PO_3 , pH 8.0), co- enzyme NAD^+, various concentrations of water- soluble 17β- estradiol and mitochondria prepared from mouse hearts according to the protocol in the section 3.8.5. The optimal concentration of physiologically active βNAD^+ required for the reaction was determined using 100 µg of mitochondria, 10 mM of E2 and various concentrations of NAD^+. The reaction mixtures (200 µl each) were pipetted into 96-well plate and optical density (OD) of generated NADH was continuously measured using Spectra Thermo reader (Tecan) at 340 nm wave length and molar extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH as indicated in the literature. The reactions were run for 30 min. at room temperature. The OD values were recorded in 1 minute intervals and the values measured inover a period of 15 minutes were taken for standard curve plotting. OD values generated by mitochondria alone were subtracted from the enzyme plus substrate measurements. The NAD concentration that resulted in the highest NADH production (i.e. 200 µM), was used for further experiments. Subsequent experiments were performed using 100 µg mitochondria, 200 µM NAD and various concentrations of 17β- estradiol in assay buffer (200µl total volume), if not indicated otherwise. Absolute estrone (E1) amounts generated during enzymatic reactions were determined using Estrone ELISA assay (Ecologiena®) according to the manufacturer's protocol except the sample preparation step. For current assay each reaction mixture was diluted 1:200 in 10% methanol and then used for E1 determination. The absolute amounts of NADH generated during enzymatic reactions were calculated from a standard curve which was obtained OD measurements of known NADH concentrations.

3.8.7. Co- immunoprecipitation of ERαLBD and His_17βHSD10

HELA cells were splitted a day before transfection and cultivated in phenol- red free Dulbecco's Modified Eagle's Medium (DMEM, Sigma- Aldrich) containing 10% steroid hormone- free fetal calf serum (cc pro). Next day, the cells were transfected with protein expression vectors pDEST27_rERαLBD, pDEST26_17βHSD10 and co- transfected with both of them using GenePorter™ 2 Transfection Reagent protocol in combination with BoosterExpress™ Reagent Kit (both systems purchased from GTS Inc.). The absence of estrogen in DMEM was critical for protein- protein interaction studies. All transfection and boosting

procedures were performed as described in the manufacturer's instructions. In 24 hours after transfection, the HELA cells expressing GST- tagged rat ERαLBD, 6x His- tagged human 17βHSD10 and co-expressing both proteins were collected and lysed in low salt lysis buffer (50 mM Tris, pH 8.0, 1% IGEPAL CA-630, 1 mM PMSF, protease inhibitor cocktail). The cells were sonicated in shot bursts and passed several times through an insulin syringe. Untransfected cells served as a negative control for further experiments. Glutathione Sepharose® 4B beads were obtained from Amersham Biosciences. Prior to usage, the beads were washed several times with ice- cold PBS and as a 50% slurry was used for further experiments. 500 µl of HELA lysates and 200 µl of washed beads were incubated overnight at 4°C. The beads were incubated with untransfected HELA lysate (negative control), with overexpressed GST_ERαLBD, with overexpressed His_17βHSD10 (negative control) and with HELA lysate that contained both recombinant proteins (GST_ERαLBD *and* His_17βHSD10). After overnight incubation, glutathione beads were washed 3-4 times with the washing buffer containing 50 mM Tris (pH 8.0), 20 mM glutathione, 1% Igepal CA-630 and protease inhibitor cocktail. The beads were dissolved directly in Laemmli buffer, denatured and subjected to protein electrophoresis. The overexpressed GST-tagged ERαLBD was detected using rabbit polyclonal anti- ERα antibody (MC-20, Santa Cruz) and 6x His- tagged 17β HSD10 was identified using rabbit polyclonal anti- 17β HSD10 antibody (a generous gift of Prof. S.-Y. Yang from NYS Institute of basic research, NY, USA). The signal of both proteins was visualized using secondary anti- rabbit IgG and developed with ECL reagent (Amersham) according to the manufacturer's recommendations.

3.9. Statistics

Statistical analysis was performed using Anova test, Student- Newman-Keuls method. The results are presented as mean values of each treatment group ± SEM. The $P<0.05$ values were considered as statistically significant and assigned as $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$. The statistical analysis was performed using *SigmaStat 32* software (SPSS Inc.).

4. Results

4.1. Hormone influence on morphological parameters of SHR rats

The substitution of young ovariectomized SHR rats with 17β- estradiol as well as selective estrogen receptor alpha agonist 16α- LE_2, effectively attenuated body weight of young ovariectomized SHRs which was comparable to sham operated rats (Fig.9). Similar effects were observed in estrogen and 16α- LE_2 substituted senescent rats. The ERβ agonist 8β- VE_2 did not influence body weight which was comparable to young ovx rats. The senescent animals, however, did not gain weight under the treatment of 8β- VE_2. Significant differences could be observed among the following treatment groups: young sham vs. ovx $p< 0.001$, sham vs. ovx+8β-VE_2 $p< 0.001$, ovx vs. ovx+ E2 $p< 0.001$, ovx vs. ovx+ 16α- LE_2 $p< 0.001$; senescent ovx vs. ovx+ E2 $p= 0.005$ (not shown), ovx vs. ovx + 16α- LE_2 $p= 0.043$ and ovx vs. ovx+ 8β- VE_2 $p= 0.039$.The number of the rats per group varied between 18 to 20 in the young SHR rat study and between 7 to 9 in senescent SHR rat study.

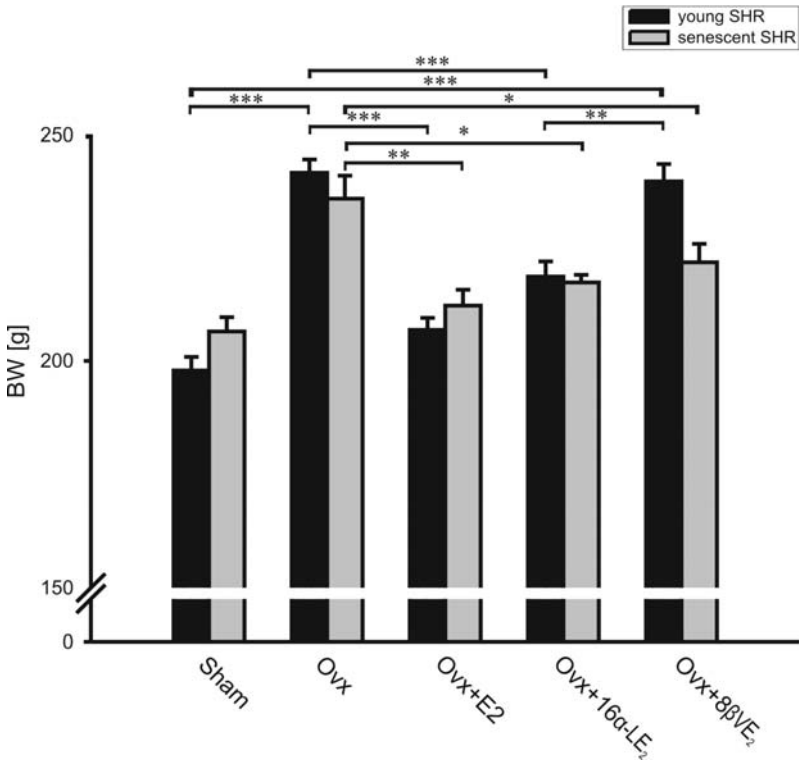


Figure 9. Absolute body weight of young and senescent SHR rats. Absolute body weight of sham operated, ovariectomized SHR treated with placebo (peanut oil), 17β-estradiol, ERα agonist 16α-LE_2 and ERβ agonist 8β- VE_2 treated rats. Solid bars represent young SHR rat groups and open bars show senescent SHR rats treatment groups.

Uterus weight represents one of the classical morphological parameters for evaluation of estrogen substitution as well as ER ligand efficacy and specificity *in vivo*. In both, young and senescent animals, uterus weight varied significantly due to oestrus cycle in sham operated SHR rats. Due to ovariectomy and resulting endogenous estrogen deprivation, the uterus weight was lower in young ovx SHR rats comparing to sham operated rats. In senescent rats, however, the ovariectomy caused only slight reduction in uterus weight. Both substances 17β-estradiol as well as 16α-LE_2 increased significantly uterus weight in young ovariectomized rats. In contrast to 16α-LE_2, uterus weight of rats substituted with ERβ agonist 8β- VE_2 was similar to the ovariectomized ones. Uterus weight of senescent SHR rats was also affected by E2 and 16α-LE_2, but not by 8β- VE_2. The removal of ovaries in senescent rats caused a significant decrease in uterus weight but residual weight was higher comparing to young ovariectomized rats. The estradiol and 16α-LE_2 substitution of senescent rats could significantly increase the uterus weight whereas ERβ agonist did not show the uterotrophic effects. The summary results of all treatment groups are shown in Figure 10.

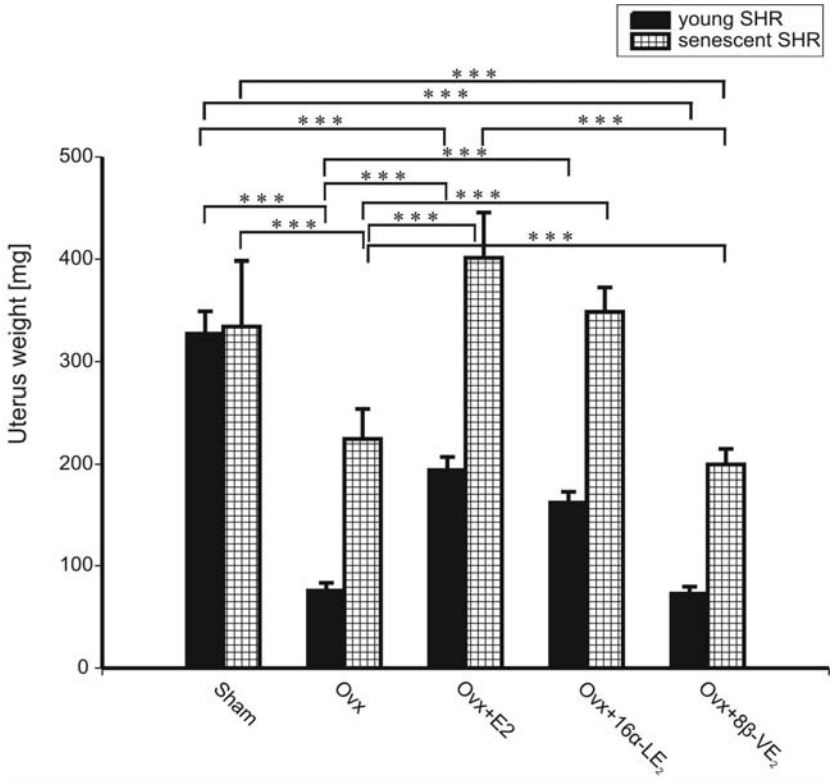


Figure 10. **Absolute uterus weight of young and senescent SHR rats**. Young and senescent SHR rats were sham operated (sham), ovariectomized (ovx) and treated with placebo (peanut oil+ ethanol), 17β- estradiol, ERα agonist 16α-LE2 and ERβ agonist 8β-VE_2. Solid bars represent mean ± SEM of young SHR rat uterus weight and the open bars show mean uterus weight ± SEM of senescent SHR rat groups. ***P< 0.001.

Significantly elevated heart weight is a morphological marker of cardiac hypertrophy. Therefore, the cardiac mass was measured and compared between both age groups and among all treatment groups in the current work. The degree of cardiac hypertrophy was estimated normalizing absolute heart weight to tibia length. Anti- hypertrophic properties of 17β-estradiol in SHR rats are well documented [87, 137]. Therefore, the treatment of ovariectomized SHR rats with 17β- estradiol served as a positive control for evaluation of 16α-LE_2 and 8β-VE_2 for their ability and potency to attenuate cardiac hypertrophy. Relative heart weight was calculated as a ratio of absolute heart weight- to- tibia length. Cardiac mass of young ovariectomized SHR rats was significantly increased compared to sham operated controls. Significant reduction of heart weight could be observed in 17β- estadiol, 16α-LE_2 and 8β-VE_2 treatment groups. Normalization of absolute heart weight versus tibia length showed that estradiol and both selective agonists could effectively reduce cardiac hypertrophy in young SHRs. Senescent rat heart weight analysis showed substantially higher degree of cardiac hypertrophy compared to young individuals (sham young 1078± 52 mg vs. sham senescent 1230± 43 mg). Neither removal of ovaries, nor substitution with estradiol or selective agonists did influence heart weight in senescent SHRs. Normalization of absolute heart weight versus tibia length provided comparable results. Summary data of absolute and relative heart weight are shown in Figure 11.

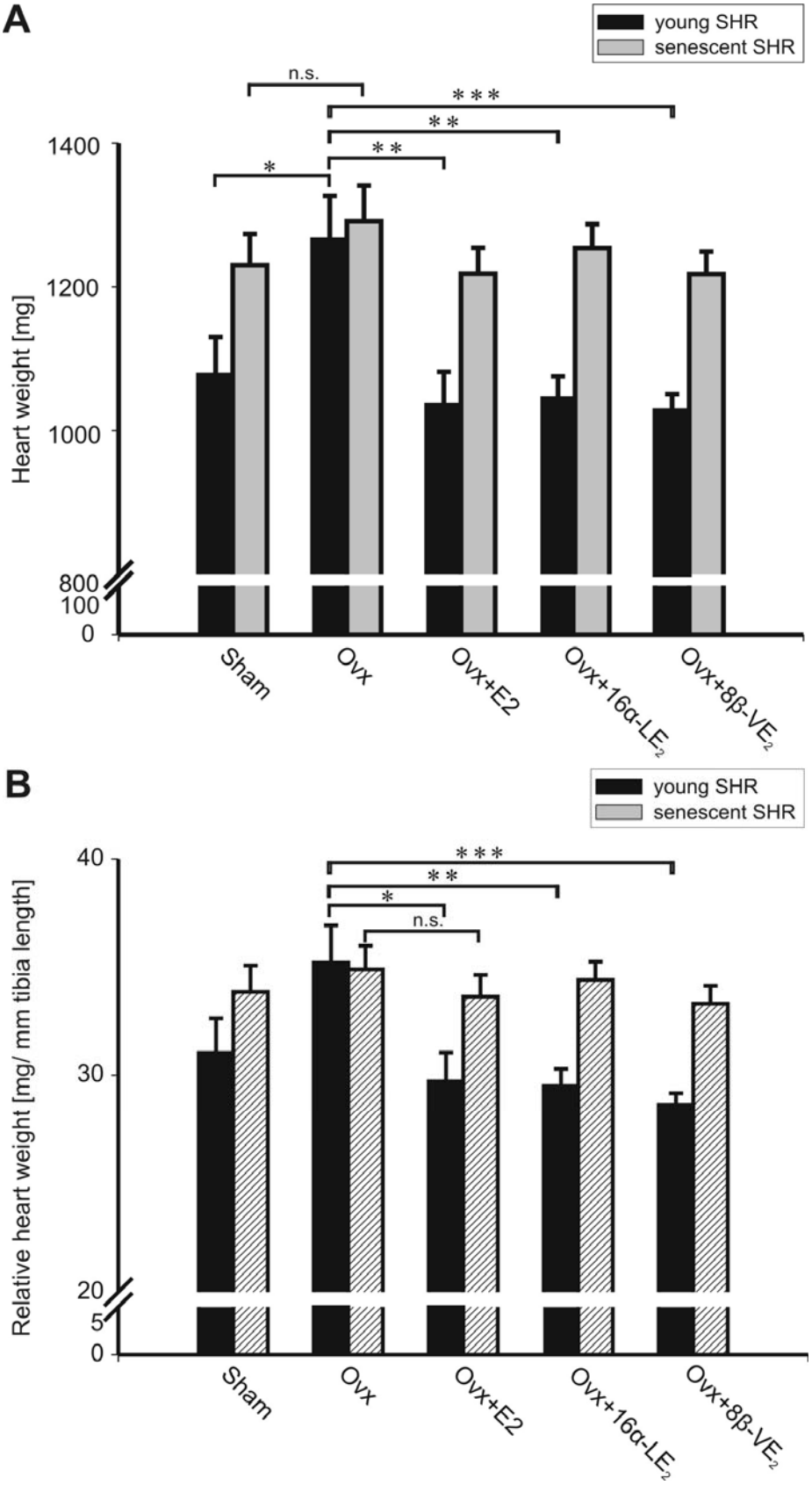


Figure 11. Absolute and relative heart weight of SHR rats. The solid bars show the mean absolute heart weight of young SHR rats (10A) and the mean of relative heart weight (10B). The absolute and relative heart weight of senescent rats is shown in Fig. 10A and 10B, respectively. No significant differences were observed between the treatment groups of senescent SHR. Each bar represents mean heart weight in mg ± SEM (n= 20 young animals per group and n= 8 senescent animals/ group, **P*< 0.05, ***P*< 0.01 and ****P*< 0.001, n.s- non- significant).

4.2. Hemodynamic measurements

Invasive hemodynamic measurements were performed as described in the *Methods*. Surprisingly, 8β-VE_2 treatment of ovariectomized young rats could significantly attenuate systolic blood pressure which was even lower than in sham rats. In senescent rats, left ventricular systolic pressure was higher comparing to young animals and remained similar in all treatment groups (Fig.12A). Heart rate (HR) of young animals was higher than that of senescent rats and was not affected by ovariectomy or by hormone treatment. Systolic aortic pressure (SAP) measurements in young SHR rats showed significant aortic pressure decrease in 8β-VE_2 substituted group. Aortic pressure in senescent rats was significantly higher comparing to in their young counterparts. This parameter was comparable among the treatment groups (figure 12B). Cardiac output (CO) was highly increased in young animals receiving 16α-LE_2 and 8β- VE_2 (67±3 and 66±2 ml/min) compared to sham and ovx rats (58±3 and 56±1 ml/min, respectively). In senescent rats, cardiac output was significantly lower compared to young animals and cardiac output values were not different among the different treatment groups (figure 12C). The results of invasive hemodynamic measurements are summarized in Table 1.

	Heart rate beats/min	Cardiac output ml/min	Systolic aortic pressure mmHg	Diastolic aortic pressure mmHg	Mean aortic pressure mmHg
Sham young	341± 5	58±2.7 ‡ *	221± 5.8 #	150± 5.7	174± 5.3 *
Ovx young	355± 7	56±1.5 § #	219± 5.7 §	147± 5.7	171± 4.8 ‡
Ovx+E2 young	363± 8	61± 2.1	210± 6.5 ‡°	138± 5.3	162± 5.4 §
Ovx+16α-LE_2 young	359± 9	67±2.8 * § °	209± 5.5 *	144± 3.2	166± 3.9 #
Ovx+8β-VE_2 young	337± 8	66± 1.9 ‡ # &	187± 3.8 # § ‡ *&	130± 5.0 &	149± 3.8 * ‡ § # &
Sham senescent	312± 13	48± 4.0	244± 13.5	170± 5.0	192± 7.4
Ovx senescent	319± 7	50± 3.4	236± 14.2	153± 6.8	181± 8.8
Ovx+E2 senescent	325± 8	52± 3.4	251± 5.1 °	162± 5.7	192± 4.3
Ovx+16α-LE_2 senescent	321± 11	50± 1.6 °	231± 12.7	153± 9.3	179± 10
Ovx+8β-VE_2 senescent	314± 10	47± 2.6 &	250± 7 &	166± 5.1 &	194± 5.0 &

Table 1. Invasive hemodynamic measurements. Significant differences (P<0.05) in young and senescent SHR rats which were sham operated (Sham), ovariectomized and treated either with placebo (Ovx) or with 17β-estradiol (Ovx+ E2), ERα agonist 16α-LE_2 (Ovx+16α-LE_2) or ERβ agonist 8β-VE_2 (Ovx+8β-VE_2). The treatment of senescent rats did not reveal any significant changes between the treatment groups whereas in SHRs, treatment with 16α-LE_2 and 8β-VE_2 resulted in significant improvement of cardiac function. The symbols next to the parameter values indicate significant differences (P<0.05) between the treatment groups of young and senescent SHRs. Each value represents mean± SEM.

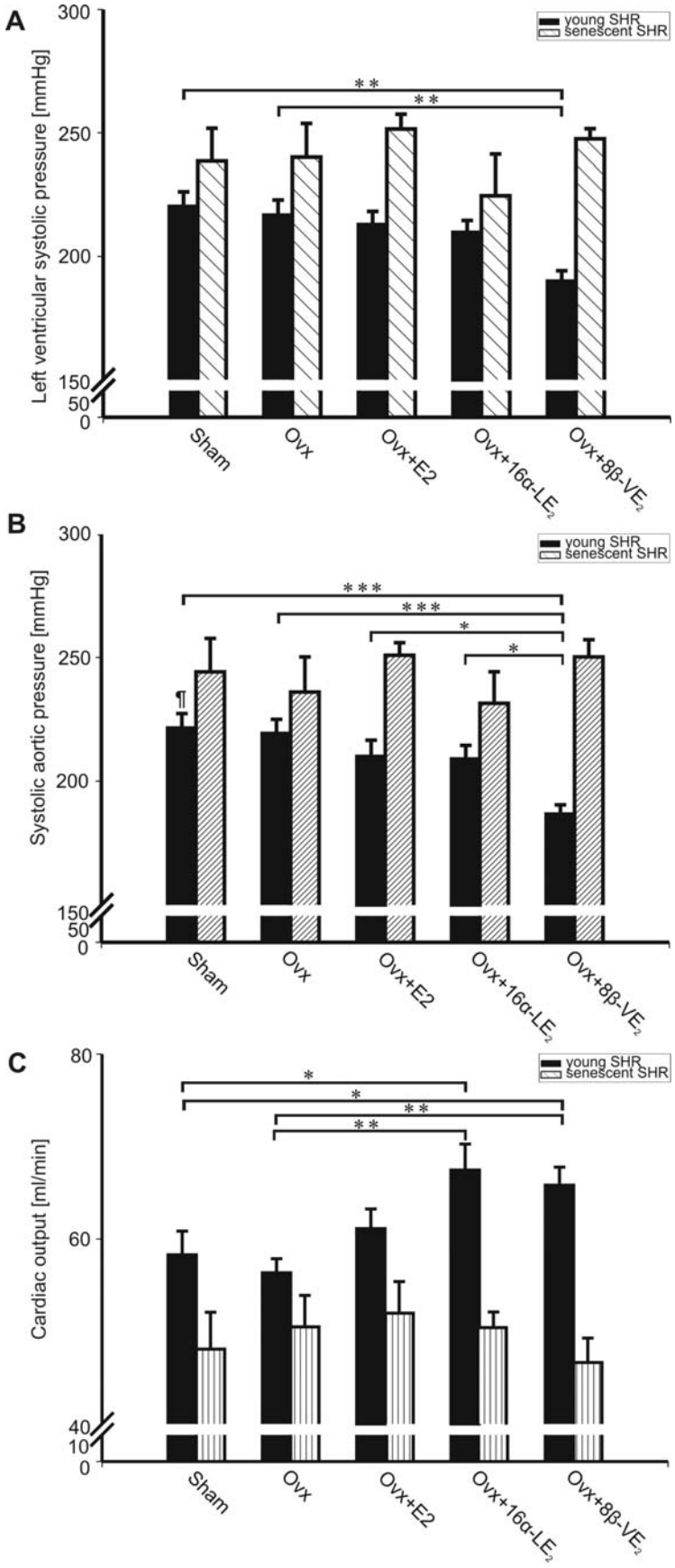


Figure 12. Representative diagrams of hemodynamic data. Left ventricular systolic pressure as well as aortic pressure could be attenuated by 8β- VE_2 in young SHR rats (Fig. 12A and 12B, respectively). Both agonists were superior in augmenting cardiac output in young SHRs (Fig. 12C) (solid bars). The treatment of senescent SHR rats with either 17β- estradiol or with agonists did not affect hemodynamic parameters (open bars). The data is presented as mean± SEM.

4.3. Estrogen, its precursors and metabolites in the plasma. Quantitative analysis

Plasma hormone level measurements in ovariectomized and estrogen- treated female SHR rats provided important information about hormone substitution efficacy. The measurements of estrogen metabolites estrone and estriol gave the first insights in estrogen metabolism dynamics in young and senescent individuals. Plasma levels of testosterone, its precursor 4- androstenedione and 5α- dihydrotestosterone were analyzed. The metabolic pathways of estrogen and androgen metabolism are schematically represented in figure 13. The first steroid hormone biosynthesis step is conversion of cholesterol to pregnenolone. From pregnenolone, steroid biosynthesis proceed either through so- called “delta- 5” pathway (17α- hydroxypregnenolone, dehydroepiandrosterone, testosterone), or through the “delta- 4” pathway (progesterone onwards). Estrogens are generated from androgens (androstenedione and/or testosterone). Irreversible reactions are denoted by a single arrowhead. Reversible reactions (double arrowhead arrows) depend on cofactor availability (e.g. the NADH/ NAD or NADP/ NADPH ratio).

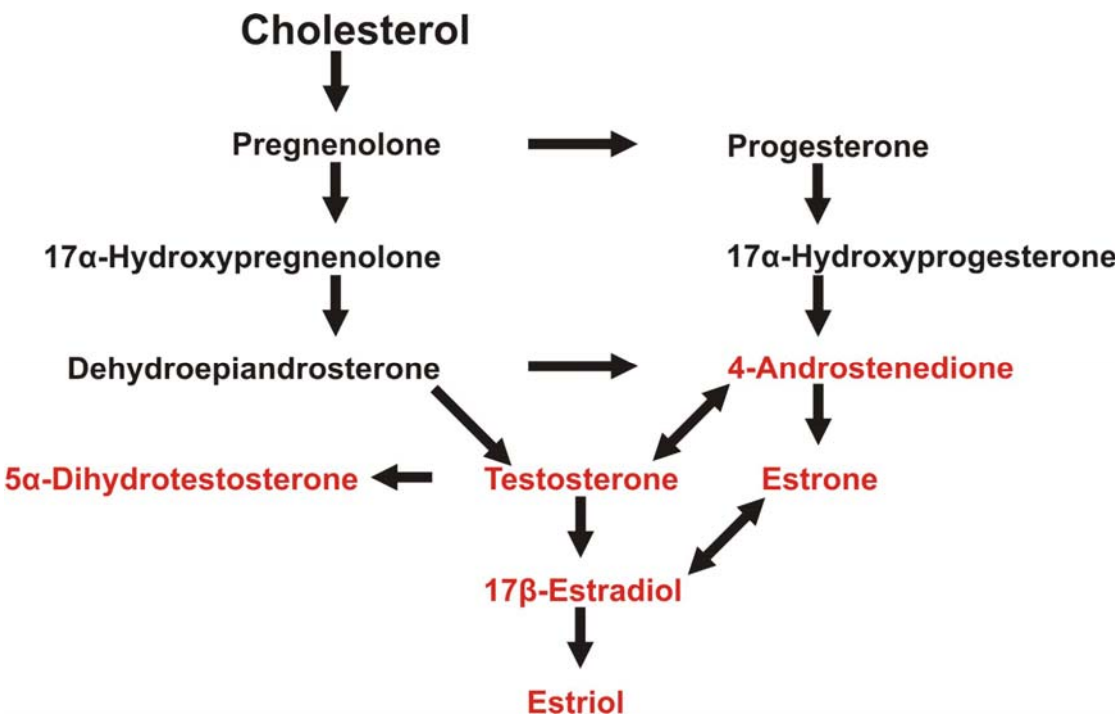


Figure 13. Estrogen and androgen metabolism pathways. The scheme shows major hormones and their intermediate metabolites. The water- soluble metabolites are omitted. The hormones highlighted in red plus free testosterone have been determined in the serum probes of young and senescent SHR rats.

Ovariectomy of young SHR rats resulted in significant reduction of endogenous estrogens and androgens compared to sham operated animals. Estrogen levels in ovariectomized rats were 4.5- fold lower comparing to sham operated rats (12.6± 3 vs. 57.2± 11.8 pg/ml, p= 0.008). Estrogen plasma levels could be elevated with daily injections of 17β- estradiol (2 µg/kg/day). Hormone treatment resulted in significantly higher plasma estrogens concentrations comparing to endogenous estrogen in sham operated animals (105.6± 36.2 vs. 57.2± 11.8 pg/ml, p= 0.002). Estrogen injections were performed 12 to 14 hours before invasive hemodynamic measurements. Estrogen levels measured in 16α-LE_2 treated rats were similar to those in sham operated animals which could be explained by its structural similarity to 17β- estradiol. Estrogen levels in 8β- VE_2-substituted rats were comparable to those in ovariectomized rats. In senescent animals, plasma estrogen levels were low in all treatment groups (sham 19.72± 1.16 pg/ml; ovx 14.0± 1.18 pg/ml) and were comparable to those of young ovariectomized individuals. Estrogen metabolites- estrone and estriol- were measured to evaluate the rates of estrogen metabolism. Plasma estrone levels in young and senescent SHR rats were similar to estrogen levels in both age and treatment groups (Fig. 14, panels A and B). Plasma estriol concentration in young sham operated animals was significantly lower comparing to senescent sham rats (78.28± 11.6 vs. 136.4± 11.0 pg/ml, p= 0.01). Ovariectomy of young animals did not alter plasma estriol levels whereas estrone levels in senescent ovariectomized rats were significantly reduced compared to sham operated individuals (136.4± 11 vs. 82.29± 11.14 pg/ml, p= 0.01) (Fig. 14, panel C). Estrogen precursor testosterone (total and free testosterone fractions), 5α-dihydrotestosterone and 4- androstenedione have been determined. Ovariectomy of young and old rats resulted in substantial decrease of all androgens measured (Table 2). The complete results of hormone measurements are summarized in Table 2.

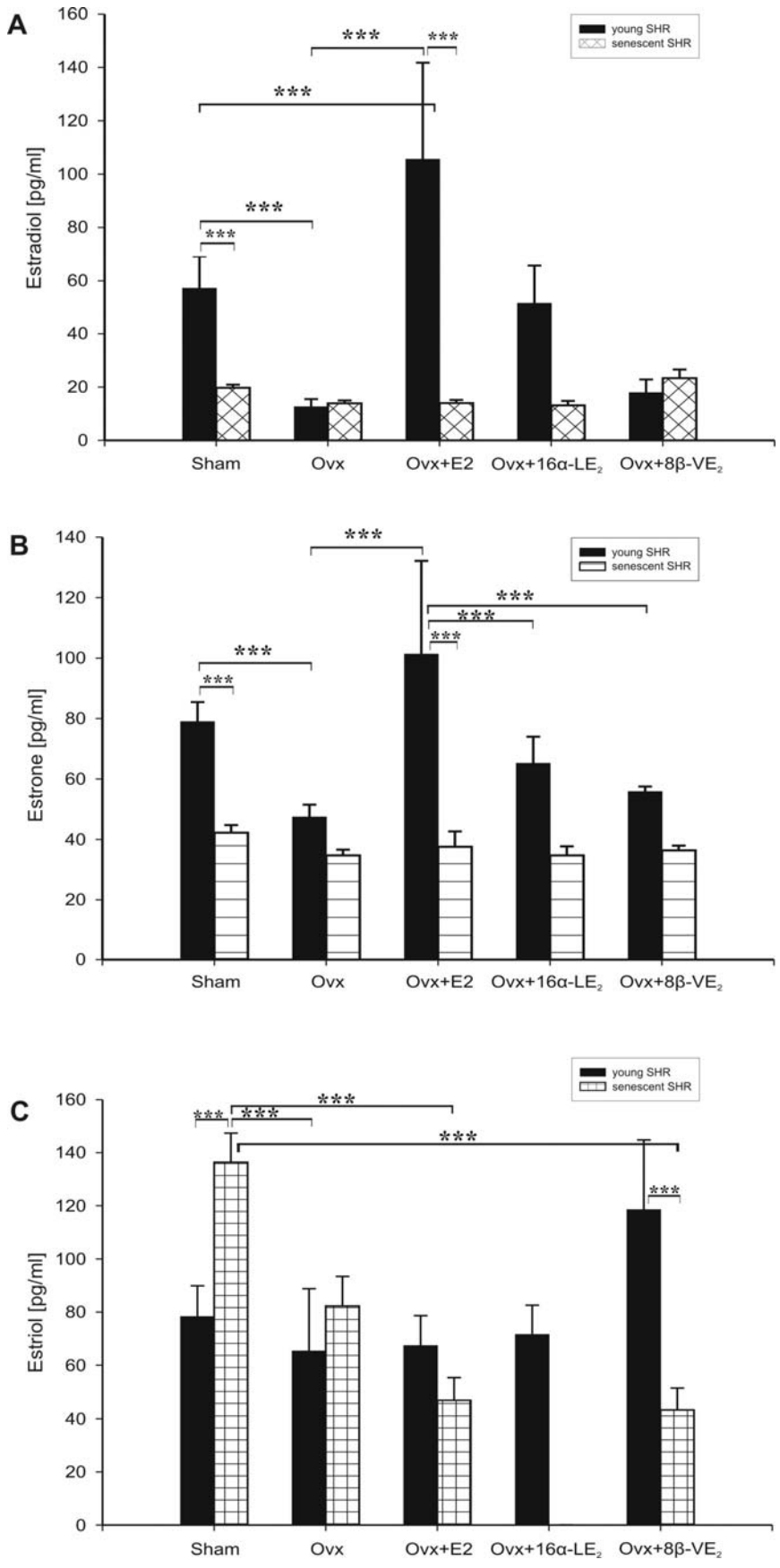


Figure 14. Estrogen, estrone and estriol plasma levels in young and senescent SHR rats. The solid bars represent the data of young animals while open bars show the results of senescent animals. Results are expressed as mean ± SEM.

Ovx+ βAg senesc.	23.31± 3.25	36.36± 1.6	43.33± 8.4*	83.4± 21.5	31.0± 7.5	0.239± 0.02	32.2± 3.6
	Estradiol [pg/ml]	Estrone [pg/ml]	Estriol [pg/ml]	4- Androstenedione [pg/ml]	Total Testosterone [pg/ ml]	Free Testosterone pg/ml	5α-Dihydrotestosterone [pg/ml]
Sham young	57.19± 11.8 # ‡ †	78.96± 6.5 * ¶	78.28± 11.6	456.8± 25.2 * # § ‡	476.7± 83.4 ¶ *	1.04± 0.17 *	106.4± 20 # *
Ovx young	12.56± 29 * #	47.4± 4.1 # ¶	65.33± 23.48	101.44± 24.3 *	35.6± 7.5 ¶	0.133± 0.01 *	35.7± 5.1 #
Ovx+E2 young	105.57± 36.2 * § ¶ †	101.3± 30.83 # § ‡	67.33± 11.33	132.9± 12.7 #	23.2± 4.0	0.156± 0.01	51.7± 4.2
Ovx+ αAg young	51.5± 14.2	65.2± 8.81 ‡	71.57± 11	161.3± 26.7 §	44.9± 8.1	0.137± 0.02	46.7± 3.7
Ovx+ βAg young	17.87± 4.96 § ‡	55.8± 1.7 §	118.5± 26.3	35.5± 18.3 ‡	16.0± 4.0	0.183± 0.02	28.4± 3.6
Sham senesc.	19.72± 1.16	42.24± 2.52 *	136.4± 11 # * §	260.4± 22.7 ¶	123.7± 16.8 § *	0.505± 0.03 ¶	35.8± 5.3 *
Ovx senesc.	13.9± 1.0	34.7± 1.92	82.29± 11.14 #	44.4± 9.6 ¶	18.3± 2.9 §	0.192± 0.02 ¶	17.8± 2.5
Ovx+E2 senesc.	14.0± 1.18 ¶	37.55± 5.1	46.75± 8.52 §	125.3± 22.6	22.0± 4.8	0.248± 0.02	26.8± 3.2
Ovx+ αAg senesc.	13.13± 1.65	34.7± 3.03	0.08± 0.03	93.6± 19.1	40.3± 17.4	0.28± 0.02	27.4± 5.1

Table 2. Hormone concentrations in plasma of young and senescent SHR rats. One way Anova analysis and Student- Newman- Keuls test. Each value represents mean pg/ml ± SEM. A *P* value of < 0.05 was considered to be statistically significant.

4.4. Myosin heavy chain (MHC) expression in the SHR rat hearts

Myosin heavy chain (MHC) isoforms- α- and β MHC, expression in small rodent heart is a dynamic process which depends on the developmental state of the individual, availability of mechanical and/ or hormonal stimuli, cardiac hypertrophy and others. In the healthy myocardium, αMHC or “adult MHC” is the major isoform whereas βMHC or “fetal MHC” which was repressed or shut off during development is reactivated in the process of hypertrophy. MHC isoform expression is also species specific, i.e. small rodents, such as adult mice and rats predominantly express αMHC whereas βMHC is the dominant isoform in bigger mammals (rabbits, pigs and humans). Therefore, the ratio between αMHC and βMHC isoforms plays a major role in the determination of cardiac contraction velocity and force generation. Additionally, the shift from α- to- β- MHC in the rodent heart is serves as a molecular marker of cardiac hypertrophy [138].

In young SHR rat hearts, αMHC was the more prevalent myosin isoform whereas in ovariectomized rat hearts the ratio α- to- β- MHC was shifted towards βMHC compared to sham operated control (1.077± 0.0652 vs. 1.739± 0.118, P=0.002). The treatment of ovariectomized rats with 17β- estradiol and ERα agonist 16α-LE_2 increased the ratio towards αMHC (1.522± 0.134 and 1.73± 0.143, respectively). The substitution of ERβ agonist 8β- VE_2 influenced little the MHC expression and α- to- β MHC ratio was similar to that of ovariectomized individuals (1.151± 0.093) (figure 15A). The hormonal treatment of senescent ovariectomized rats was not as effective as in young animals. Though a slight upregulation of αMHC was observed in estradiol and 16α-LE_2 treatment groups as well as in the sham group, it did not reach the significant levels. The βMHC protein levels in ovariectomized rats and 8β-VE_2 treatment group remained similar to βMHC levels in the same groups of young SHR rats (figure 15B).

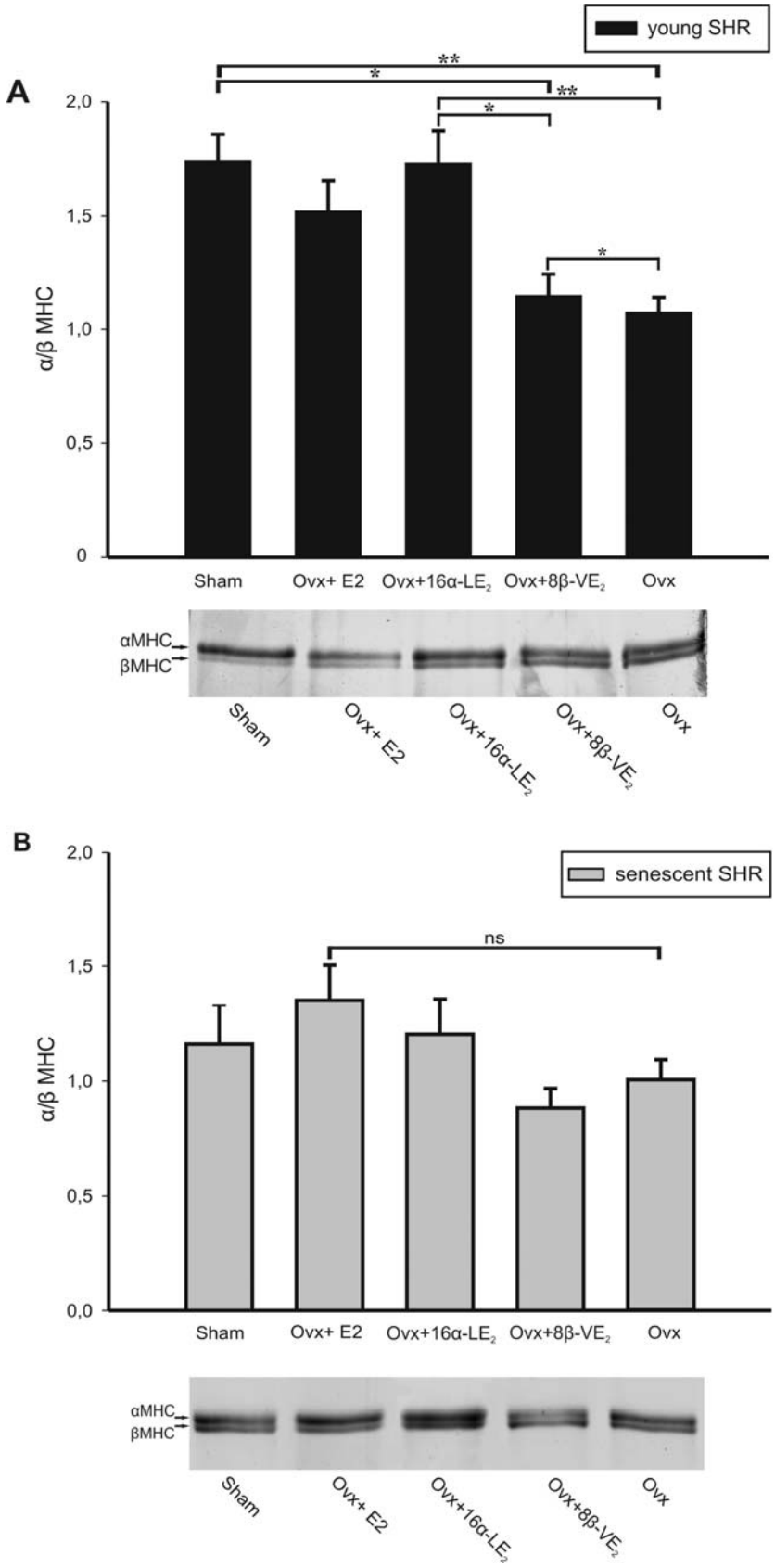


Figure 15. α/β myosin heavy chain (MHC) protein expression in young and senescent SHR rat hearts. The results of native protein gel densitometric analysis as well as representative MHC gels of young (panel A) and senescent (panel B) SHR rats. The bars show mean value of α-to-βMHC ratio, the error bars indicate SEM. $P< 0.05$ was considered as statistically significant.

4.5. Cardiac estrogen receptor alpha and beta protein expression in young and senescent SHR

ERα and ERβ were detected in the rat heart and protein expression analysis revealed that in young SHR rat hearts, ERα expression was comparable among the treatment groups (figure 16B). ERβ expression in the heart of young SHR was comparable between all treatment groups (figure 17B). The basal ERα expression was significantly lower in senescent SHR hearts comparing to young SHR hearts, comprising 0.17± 0.04 arbitrary units in senescent sham versus 0.49± 0.09 in young shams ($p< 0.05$). The ovariectomy of senescent rats did not influence the expression levels of ERα, whereas ERβ expression was non-significantly elevated in the same hearts (figures 16 and 17, panel C). Upon estrogen substitution, ERα was significantly upregulated comparing to ovariectomized individuals (0.41± 0.02 vs. 0.13± 0.02, $p= 0.009$) and comparable to the receptor expression levels in young hearts. The substitution with ERα agonist 16α-LE_2 partially restored ERα expression in senescent hearts. In contrast to ERα, ERβ expression was not increased upon treatment with 16α-LE_2. Selective ERβ activation via 8β-VE_2 revealed no changes in cardiac ERα in senescent hearts and the receptor levels were comparable with those of sham and ovx groups. ERβ protein expression levels were partially restored upon 8β-VE_2 treatment compared to 16α-LE_2 treatment but did not reach the significant levels. The statistical analysis of ERα and ERβ protein expression are summarized in panels A in the figures 16 and 17.

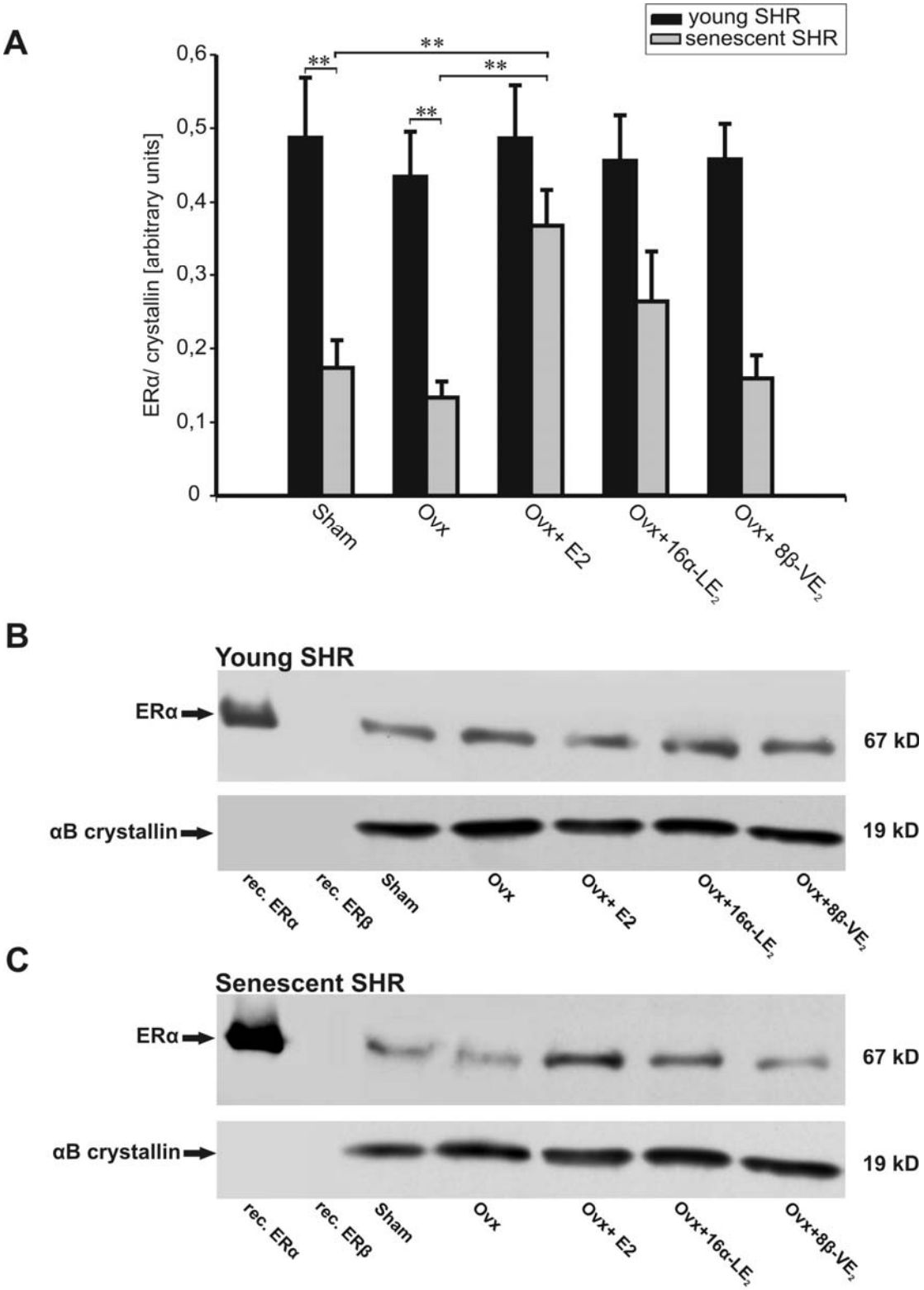


Figure 16. ERα protein expression in young and senescent female SHR rat hearts. The results of densitometrical analysis are represented in panel A as mean values and the error bars comprise standard error of the mean (SEM). The representative western blot of ERα expression in young hearts is shown in panel B whereas the pattern of the receptor expression in senescent hearts is shown in panel C. A *P* value of < 0.05 was considered to be statistically significant.

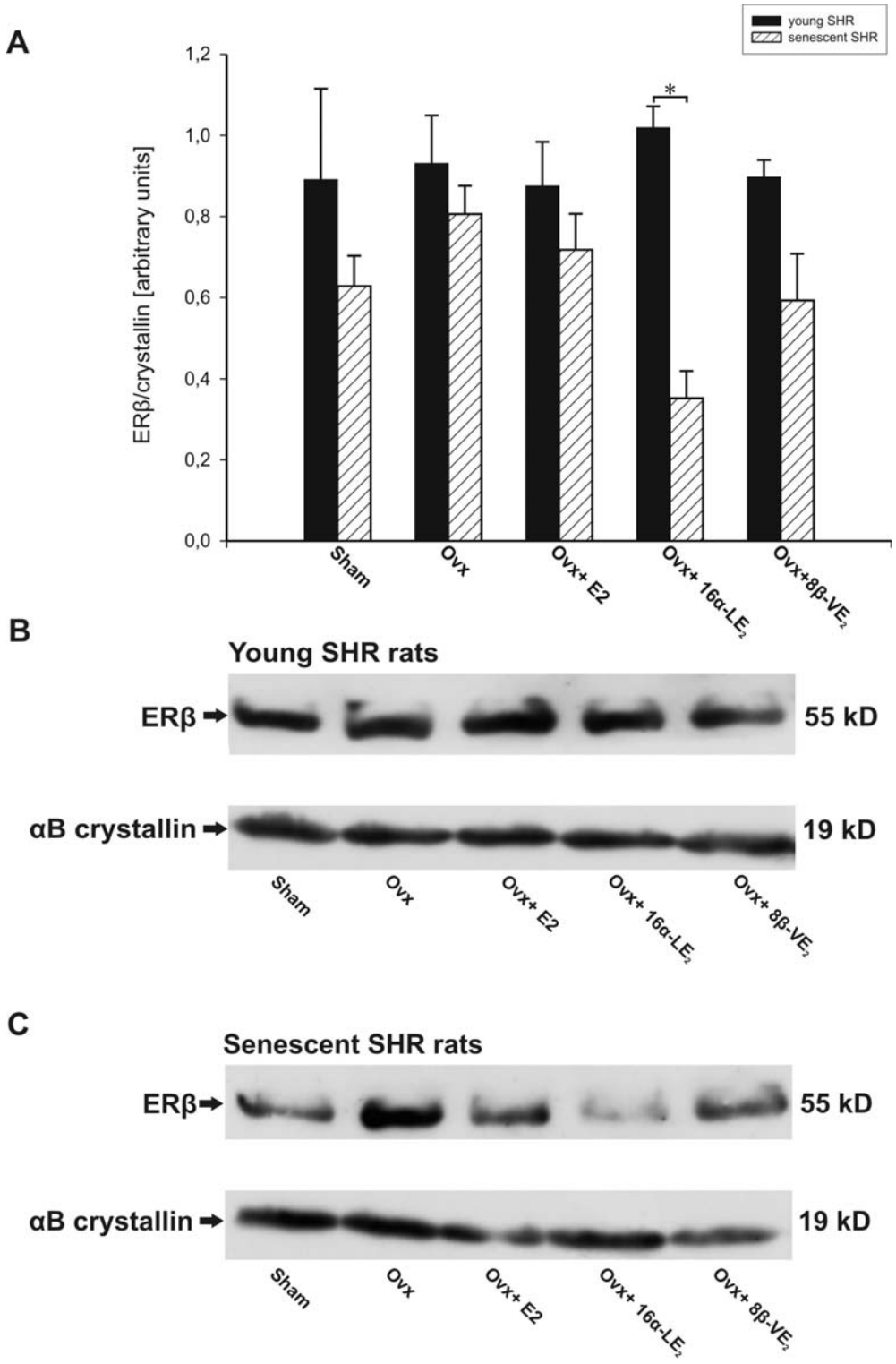


Figure 17. ERβ protein expression in young and senescent female SHR rat hearts. The statistics of ERβ expression analysis is shown in panel A as mean values, the error bars comprise standard error of the mean (SEM). The representative western blot of ERβ expression in young rat hearts is shown in panel B whereas the receptor expression in senescent hearts is shown in panel C. A *P* value of < 0.05 was considered to be statistically significant.

Estrogen receptor expression analysis in the rats showed differential ER expression in the cardiovascual system: ERα was highly expressed in the heart

but was not detectable in the aorta. ERβ could be detected in both, the heart and in the aorta of SHR rats as shown in figure 18.

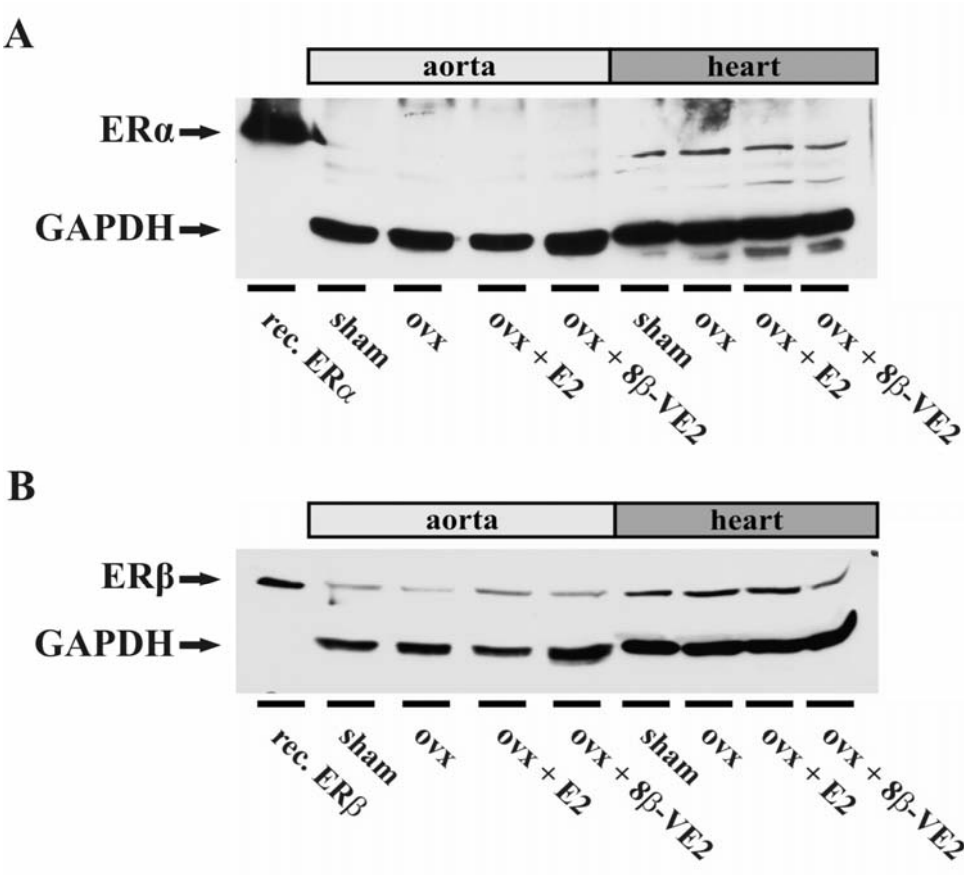


Figure 18. ERα and ERβ protein expression in rat aorta and heart. Estrogen receptor alpha could be detected in the heart but was almost undetectable in the aorta as shown in panel A whereas estrogen receptor beta could be detected in both, the aorta and in the heart (panel B).

4.6. Protein expression levels of SERCA 2, phospholamban and phosphorylated phospholamban

Cellular calcium plays a central role in cardiac muscle contraction. In relaxed muscle, calcium ions (Ca^{2+}) are stored in the sarcoplasmic reticulum (SR). Sarcoplasmic Reticulum Calcium ATPase 2 (SERCA2) is an ATP- driven pump in the cardiac myocytes which lowers intracellular Ca^{2+} concentration to less than 1 μM and plays an active role in muscle contraction/ relaxation. Phospholamban (PLB) is another calcium handling protein which, when unphosphorylated, inhibits SERCA2 via direct protein- protein interaction. In the phosphorylated state, PLB dissociates from SERCA2 and SERCA2 becomes functionally active. Therefore, overall levels of PLB as well as the degree of its active (i.e. phosphorylated) fraction (pPLB) serve as indirect parameter for SERCA2 functionality. In current

work, protein expression analyses of SERCA2, PLB and pPLB were performed to check whether estrogen deprivation and treatment with 17β-estradiol, 16α-LE_2 or 8β-VE_2 regulate protein expression levels of SERCA2 and phospholamban or influence phospholamban phosphorylation state. These data could help to explain the results obtained during invasive hemodynamic measurements. SERCA2 protein levels were non- significantly decreased in ovariectomized comparing to sham operated young rat hearts. Substitution with estrogen and treatment with 16α-LE2 did not influence SERCA2 expression compared to ovariectomized rats (figure 19, panel A). Lower SERCA2 protein expression levels were observed in senescent hearts. The 8β-VE_2 treatment resulted in moderate and non- significant increase in SERCA2 expression. SERCA2 expression analysis showed overall lower protein levels in senescent hearts comparing to young ones. Except for a slight increase in senescent ovariectomized rat hearts and decrease in 8β-VE_2 treated rats, SERCA2 cardiac protein levels were homogeneous in all treatment groups (figure 19, panel A). Statistically significant decrease in SERCA2 expression was bserved in senescent 8β-VE_2 treatment groups compared to the same treatment group of senescent SHRs. Densitometric analysis of the western blots as well as representative SERCA2 blots are shown in figure 19, panels B and C.

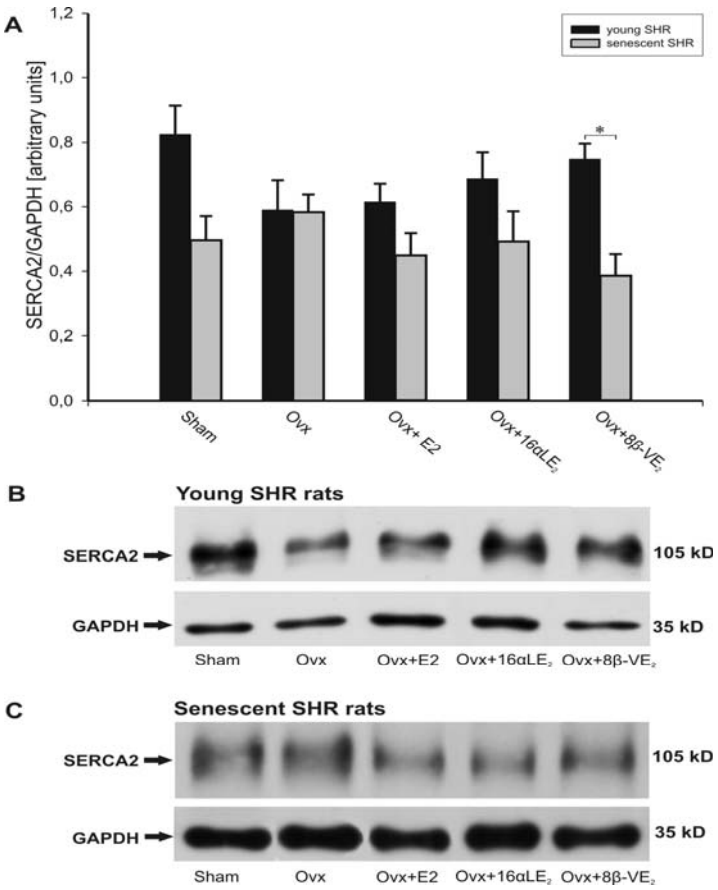


Figure 19. SERCA2 protein expression in young and senescent SHR rat hearts. The data of densitometric analysis are shown in panel A. The representative western blot of SERCA2 protein expression in young SHR rat hearts is shown in panel B. The western blot of SERCA2 expression in senescent hearts is shown in panel C. Each bar represents mean± SEM.

Total PLB levels were slightly higher in senescent SHR hearts comparing to young individuals (see figure 20, panel A). No increase in PLB expression could be observed in senescent ovariectomized rats, as well as in estrogen substituted individuals. Substitution with 16α-LE_2 or 8β-VE_2 slightly reduced PLB expression in senescent hearts (figure 20, panel C) whereas the same treatment did not influence phospholamban expression in young hearts as shown in figure 20 panel B.

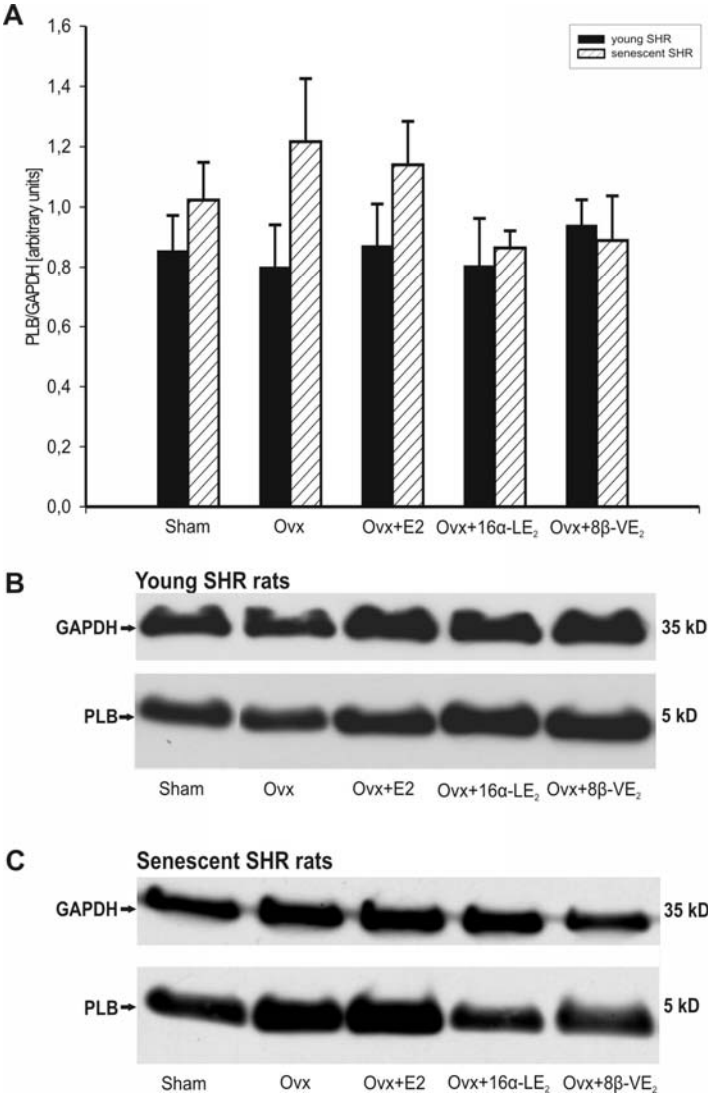


Figure 20. Phospholamban (PLB) expression in young and senescent SHR rat hearts. The data of densitometric analysis is shown in panel A. The representative western blot of phospholamban protein expression in young SHR rat hearts is shown in panel B. The western blot of PLB expression in senescent hearts is shown in panel C.

Phosphorylated phospholamban (pPLB) fraction was comparable between both age groups and among the treatment groups. Slightly higher pPLB amounts could be detected in senescent hearts and in the hearts of young sham operated rat hearts. The data of pPLB western blot densitometric analysis is presented in figure 21, panel A. Representative western blot of pPLB in young hearts is shown in panel B, whereas pPLB expression western blot of senescent hearts is presented in figure 21 panel C.

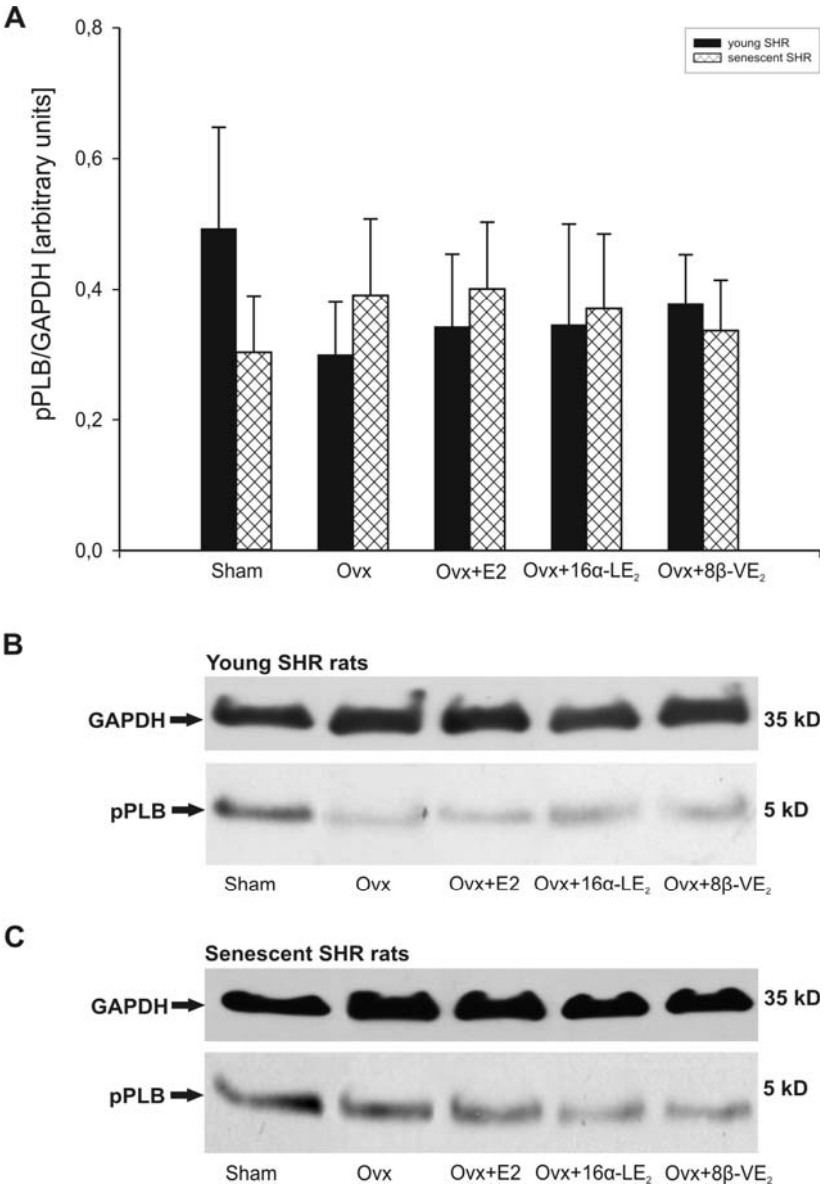


Figure 21. Phosphorylated phospholamban (pPLB) expression in young and senescent SHR rat hearts. The data of densitometric analysis is shown in panel A. The representative western blot of phospholamban protein expression in young SHR rat hearts is shown in panel B. The western blot of PLB expression in senescent hearts is shown in panel C.

4.7. Neonatal Heart cDNA library screening for protein- protein interaction partners of rat ERα ligand binding domain (LBD) (Bacterial Two Hybrid system)

As already discussed in *the Introduction,* estrogen receptors act via three distinct mechanisms: 1) genomic mechanism, where ERs act as transcription factors and can regulate target gene expression; 2) non- genomic action, where estrogen receptors can influence target protein function but not its expression and 3) can regulate protein function via direct estrogen receptor interaction with other proteins. Genomic and non- genomic estrogen receptor action mechanisms have been extensively studied in the heart whereas identification of novel and, possibly, cardiac specific protein- protein interaction candidates for ERs is a new

field. Identification and functional relevance of novel ERα protein- protein interaction partners in the heart was the main goal this project. For this purpose, BacterioMatch Two- hybrid system was used. The principle of BacterioMatch Two- hybrid system is presented in great detail in *Materials and Methods,* section 3.8. Resulting positive colonies were grown on the LB agar plates containing carbenicillin (maximal possible concentration) and expressing lacZ protein (blue/ white selection). The strength of the protein- protein interaction correlated with the blue colour intensity of the colonies, i.e. the dark blue colonies were regarded as the ones containing the gene of a protein with the strongest protein- protein interaction potential. Figure 22 shows the final colony screening procedure: positive as well as negative controls along with one of the interaction candidate gene later identified as H. sapiens 17β- hydroxysteroid dehydrogenase type 10.

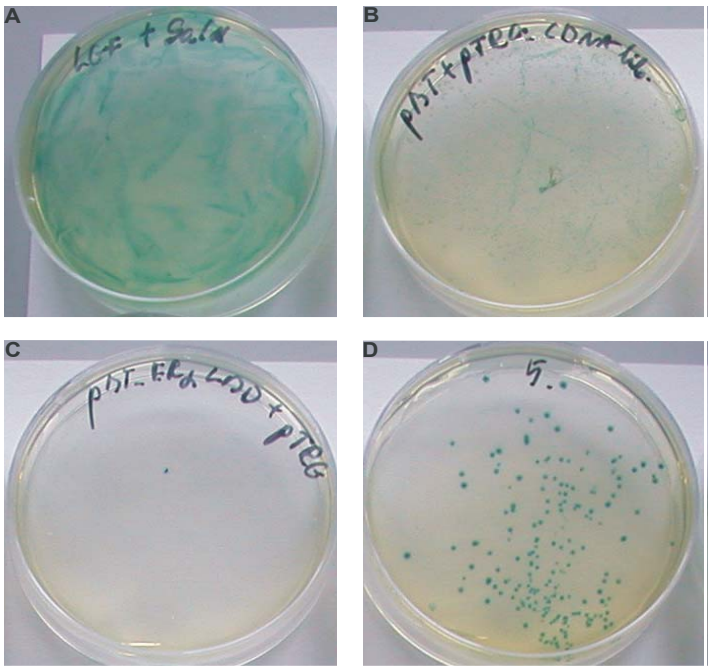


Figure 22. Positive and negative bacterial colonies obtained after embryonic human heart cDNA library screening using BacterioMatch Two- Hybrid system. The reporter strain competent cells were cotransformed with control plasmids and bait vector (pBT) containing rat ERα ligand binding domain as well as cDNA library inserted into target vector (pTRG). Positive control is shown in picture **A**. The control plasmids pBT_LGF and pTRG_Galα were cotransformed into reporter strain cells. Colonies grown on the LB agar with carbenicillin are dark blue. The figures **B** and **C** represent negative controls of the same experiment. In the figure B the backbone pBT vector was cotransformed with pTRG vector containing cDNA library, whereas in the second negative control (section C) the recombinant bait vector was cotransformed with "empty" pTRG. Both negative controls demonstrate the ability of recombinant vectors to induce alone the expression of reporter genes. The picture **D** shows one of the positive clones obtained after cotransformation of reporter strain cells with pBT_ERαLBD and pTRG_cDNA library vectors. The clone #5 later was identified as H.sapiens 17β-hydroxysteroid dehydrogenase type 10.

During the screening procedure, about 1 million cDNAs from a human heart cDNA library were screened. For further identification and analysis, 120 colonies were chosen. Blue colour intensity and resistance to antibiotics served as major selection criteria for further colony analysis. The colonies that were grown on the carbenicillin plates and those that had the most intense blue colour, were further cultivated in LB medium containing tetracycline and, to avoid artefacts, the same colonies were grown on LB agar plates containing tetracycline as well as X- Gal. Plasmid DNA extraction was done only from the colonies that were grown in both, LB medium *and* LB agar plates, and had the intense blue colour. Plasmid DNA was extracted and 120 plasmids were sequenced using pTRG forward primer (5'- TCC GTT GTG GGG AAA GTT ATC-3'). Sequence similarity search was performed using Basic Local Alignment Search Tool (BLAST) in the homepage of National Centre of Biotechnology Information (http://www.ncbi.nlm.nih.gov). The data were summarized in the table. Table 3 demonstrates the example of two candidate protein short characteristics.

Seq. Nr.	Clone #	Genebank acc. Nr. and link	Notes	LacZ staining	Homology
AVJ00060 3..822	**5**	>gi|4758503|ref|NM_004493.1| Homo sapiens hydroxyacyl-Coenzyme A dehydrogenase, type II (HADH2), mRNA Length = 990 gi|3116433|gb|AF035555.1| Homo sapiens 17beta-hydroxysteroid dehydrogenase type 10/short chain L-3-hydroxyacyl-CoA dehydrogenase (HSD17B10/SCHAD) mRNA, complete cds; nuclear gene for mitochondrial product Length = 973	No LXXLL	intense	99%
AVJ00069 60..301	**18**	>gi|13435358|ref|NM_001923.2| Homo sapiens damage-specific DNA binding protein 1, 127kDa (DDB1), mRNA Length = 4221	Contains 1 LXXLL motive	intense	99%

Table 3. The sequencing data of cDNA library positive clones. The table contains data about protein identity, the availability of LXXLL motive, blue colour intensity of positive colonies and homology between test and reference sequences.

Candidate genes for further analyses were chosen according to the following criteria: intensity of blue colour of the bacterial colony and/or the availability of LXXLL motive characteristic for estrogen receptors, the availability of the candidate protein in heart, its localization in the cell, function and functional

relevance to estrogen receptor function. The following candidate proteins were chosen for further analysis:

1. 17β-hydroxysteroid dehydrogenase type 10 (17βHSD10);

2. Bladder cancer associated protein 10 (BLCAP10);

The following strategy for general characterization of protein- protein interactions was implied: the identification of candidate proteins in heart (western blot), their localization profile (immunocytochemistry) and/or co-localization with estrogen receptor alpha in cardiac myocytes, direct interaction with estrogen receptor alpha (co-immunoprecipitation, GST pull-down assay), protein function and its functional interplay with ERs.

4.8. Localization of 17β- hydroxysteroid dehydrogenase 10 in the heart

One of the most interesting protein- protein interaction candidates is 17β-hydroxysteroid dehydrogenase type 10 (17βHSD10)/17-ketosteroid reductase. This protein represents a big family of dehydrogenase- oxidoreductase enzymes. Human 17β- HSD10 has initially been described as multifunctional mitochondrial short chain L-3 hydroxyacyl- CoA dehydrogenase (SCHAD) which maps at chromosome Xp11.2 [139] and is composed of four identical subunits of total molecular mass of 108 kD [135, 136, 140]. In addition to the functions mentioned above, this enzyme harbours alcohol dehydrogenase activity. 17β- HSD10 is NAD^+ dependent dehydrogenase and catalyzes the interconversion between active and inactive forms of estrogens and androgens. The major substrates of 17βHSD10 are 3α- androstanediol (adiol) and 17β- estradiol. The enzyme catalyses the conversion of inactive androgen, adiol, to the physiologically active form of testosterone, dihydrotestosterone. The second major substrate of 17β-HSD10 is 17β- estradiol, which can be converted to less potent estrogen, estrone (inactivation of estradiol). The unique feature of 17βHSD10 is its mitochondrial localization. Protein expression in the rat heart mitochondria was shown by western blot analysis (figure 23).

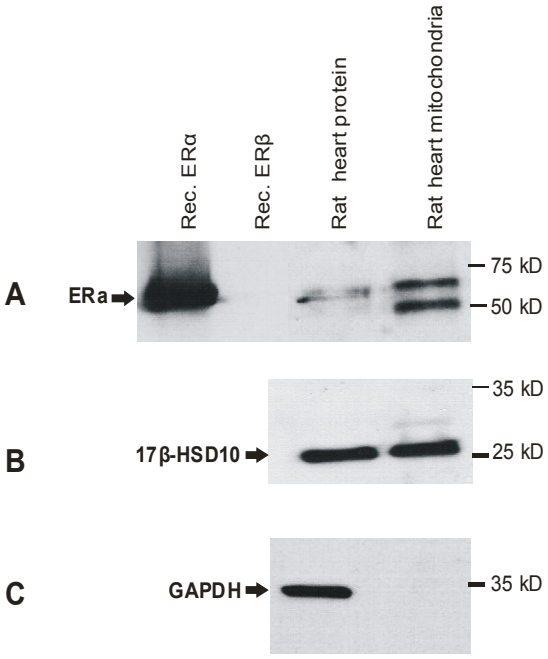


Figure 23. Estrogen receptor alpha (ERα) and 17β- dyroxysteroid-dehydrogenase type 10 (17β-HSD10) localization in rat heart protein lysate and mitochondrial fraction. Both, ERα (**A**) and 17β-HSD10 (**B**) could be detected in heart protein lysate as well as mitochondrial fraction. GAPDH (**C**) was detectable in heart protein but was absent in mitochondria. In addition to the full-length estrogen receptor (67 kD band), truncated form (50 kD) also was present in rat heart mitochondria. Recombinant estrogen receptors α and β (**A**) served as positive and negative controls for anti- ERα antibody, respectively.

Co-localization analysis of ERα and 17βHSD10 was performed in neonatal rat cardiac myocytes. Because of the antibody "incompatibility" (both, anti- ERα as well as anti- 17βHSD10, are rabbit polyclonal), the mitochondrial protein cytochrome oxidase I (COX I) mouse monoclonal antibody was used. The cellular co-localization study was performed with the help of double staining for ERα– COXI and 17βHSD10- COXI. Since COXI is expressed only in mitochondria, this protein was used as mitochondrial marker. Immunostaining experiments showed that 17βHSD10 was present only in the mitochondria whereas estrogen receptor alpha could be detected in both, nuclei and mitochondria. Minor fraction was also detected in the cytosol. To avoid artifacts in the current study, estrogen receptor alpha specific antibody was extensively tested using estrogen- receptor alpha deficient mouse liver sections to determine antibody specificity for its antigen. As shown in figure 24, mouse liver staining using specific antibody against ERα resulted in nuclear staning which is typical for ERα and this staining was absent in ERα- defficient mouse liver sections.

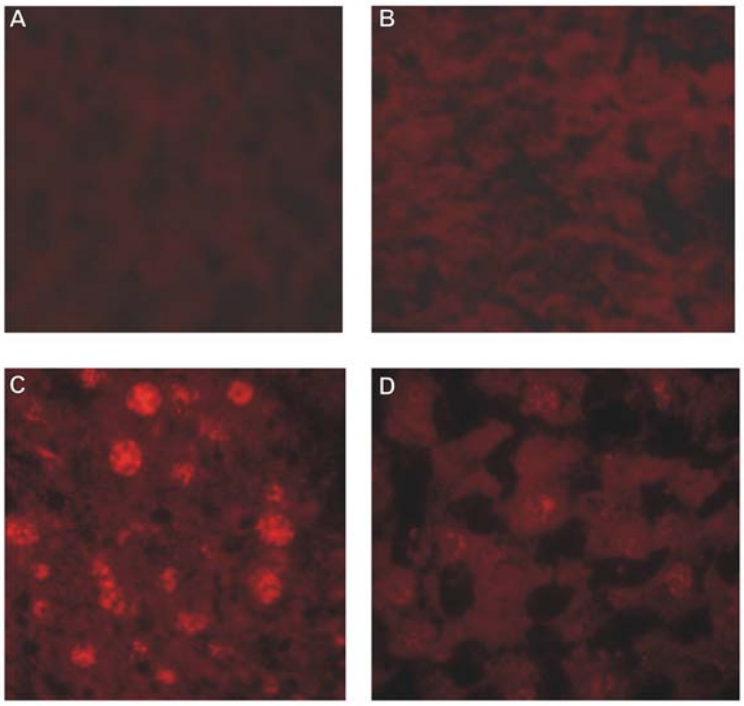


Figure 24. ERα specific antibody test using ERα deficient mouse liver sections. Rabbit polyclonal antibody against ERα (ER21) was tested using liver sections from ERα deficient (ERKO) mice and their wild- type littermates. Liver sections of wild- type (A) and ERKO (B), stained only with secondary goat anti- rabbit antibody conjugated with AlexaFluor 594 dye, served as negative control. Primary rabbit anti- ERα (ER21) and secondary goat anti- rabbit AlexaFluor 594 antibodies were used for immunostaining of wild type (C) and ER- deficient mouse liver speciments (D). Immunostaning of wild type mouse tissue resulted in nuclear staining pattern which was absent in ERα- deficient mouse liver sections.

Nuclear localization of estrogen receptors is well documented whereas mitochondrial localization of estrogen receptor alpha was described only recently. Despite published data about mitochondrial localization of both estrogen receptors, estrogen receptor beta could not be detected in the mitochondria during this study. Confocal images shown in figure 25 demonstrate immunostaining of estrogen receptor alpha, COXI and 17βHSD10 in neonatal cardiac myocytes. The merge pictures show colocalization of either COXI- ERα, or COXI-17βHSD10.

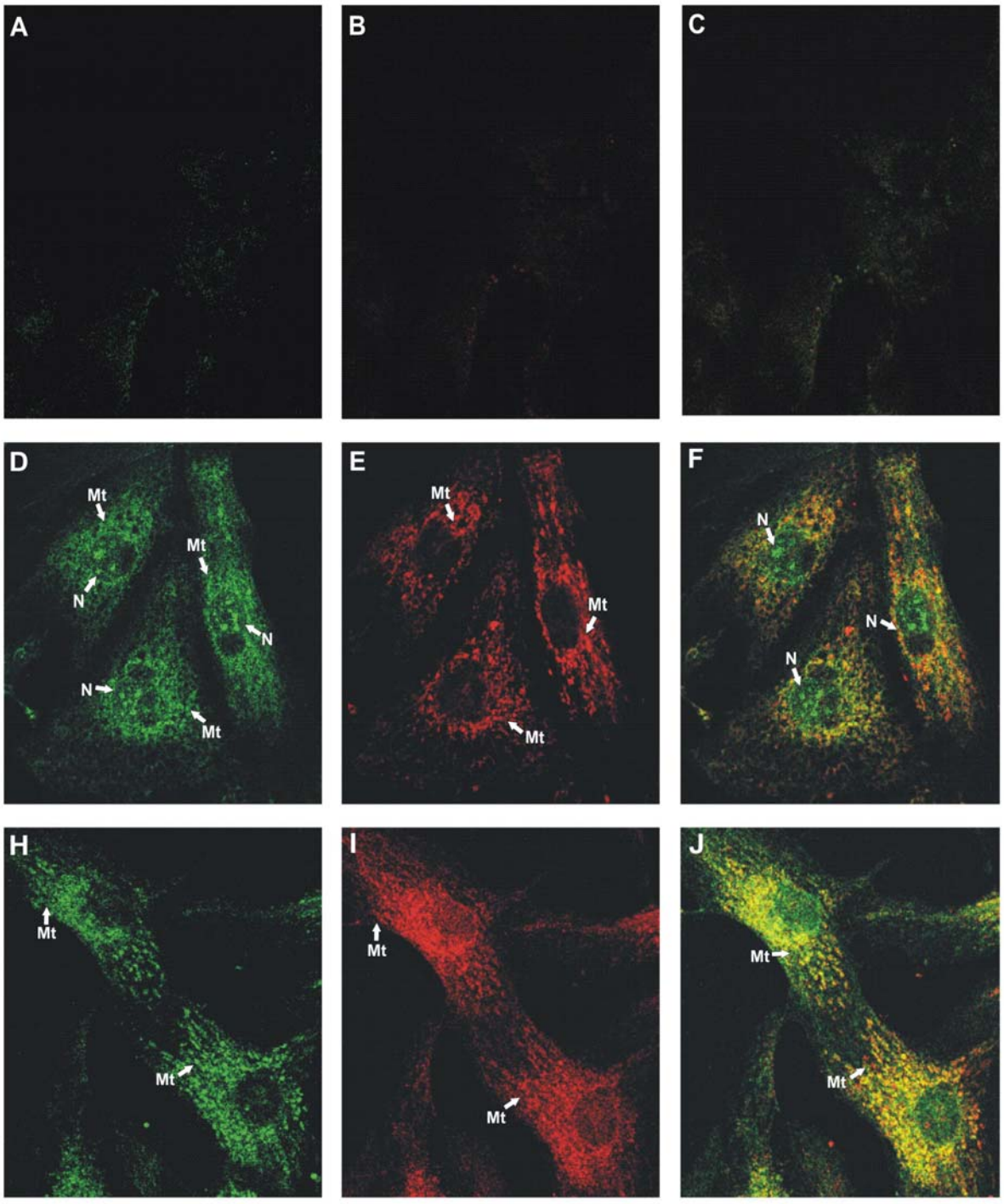


Figure 25. ERα and 17β-HSD10 co-localization in rat neonatal cardiac myocyte mitochondria. ERα intracellular localization was detected using rabbit polyclonal antibody (D). The receptor could be detected in the nuclei (N- nuclei in picture D) as well as in the cytosolic fraction. The counterstaining of the cells with anti- cytochrome oxydase I antibody (E) showed the mitochondria distribution within the cells (Mt- mitochondria). The merge picture (F) shows the overlap of estrogen receptor mitochondrial fraction and COXI signal. The double staining of 17β-HSD10 (H) and COXI (I) was performed. The merge of both signals revealed the mitochondrial (Mt) localization of both proteins (J). The secondary antibodies were used as negative controls (A- anti- rabbit; B- anti- mouse, C- merge).

4.9. ERαLBD and 17β- HSD protein- protein interaction (GST pull- down assay)

The direct protein- protein interaction of ERα and 17β-HSD10 was verified by co-immunoprecipitation assay. The ERα ligand binding domain and 17βHSD10 cDNAs were inserted into entry vector using Gateway technology (Invitrogen) and subsequently integrated into expression vectors. Due to poor transfection efficacies (2 to 5%), neonatal cardiac myocytes are in general not suitable for protein overexpression experiments. For this purpose, HELA (human cervix carcinoma) cells were transfected resulting in high expression of N- terminally tagged GST-ERαLBD and His-17β-HSD10 proteins. The generation of expression vectors as well as co- immunoprecipitation procedures are described in details in *Materials and Methods* (section 4.2). GST pull- down assays were performed on HELA cells co-transfected with the indicated expression vectors. As a negative control, untransfected HELA were used. The cells were lysed 24 hours after transfection and incubated overnight at 4°C with glutathione beads. Next day, glutathione beads were washed, dissolved in Laemmli buffer and subjected to SDS electrophoresis. Both recombintant proteins were visualized using anti- ERα (MC20) and anti- 17βHSD10 antibodies. The results of typical GST pull- down assay are presented in figure 26.

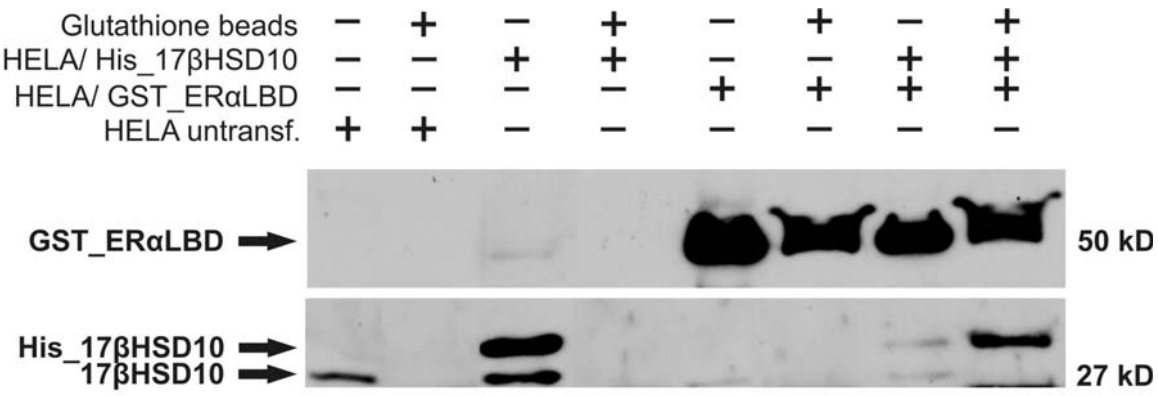


Figure 26. GST pull-down assay Co- immunoprecipitation of rat ERα ligand binding domain with 17β-HSD10.

GST_ERαLBD fusion protein but not proteins of untransfected HELA cells or histidine tagged 17β-HSD10 protein could be precipitated with glutathione beads. Co- transfected cells efficiently expressed both, GST- tagged ERαLBD and histidine- tagged 17β-HSD10. Anti- ERα antibody recognized only overexpressed GST-ERαLBD but not endogenous receptor whereas anti- 17β-HSD10 antibody

reacted with both, tagged and endogenous 17β-HSD10. The immunocomplex consisting of both tagged proteins could be purified and visualized by specific antibodies. Endogenous 17β-HSD10 was not detectable in the precipitated protein complex. This could be probably explained by the fact that endogenous HSD10 was expressed in smaller amounts comparing to His_17βHSD10 and, therefore, not detectable by the antibody.

4.10. 17β- hydroxysteroid dehydrogenase 10 enzymatic assay

17β-HSD10 oxidizes (inactivates) 17β- estradiol to estrone as shown in figure 27.

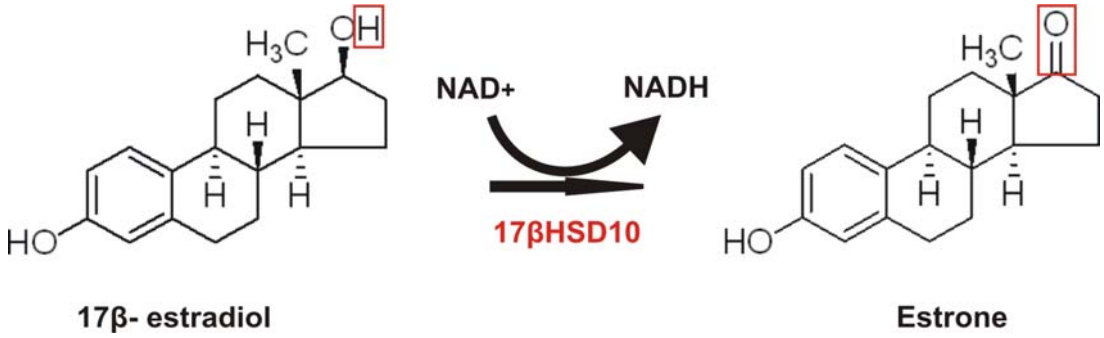


Figure 27. 17β- estradiol inactivation by 17β- HSD type 10.

The enzyme converts 17β- estradiol to physiologically less active metabolite estrone. The oxidized form of coenzyme NAD^+ is an electron acceptor, which is reduced to NADH upon electron acceptance. Previous studies performed by Yang S.-Y. and colleagues showed that 17β-HSD10 exerts its oxidative function in alkaline environment and its maximal reaction velocity is achieved at pH 8.0. This feature and unique mitochondrial localization of 17β- HSD10 helped to develop an enzymatic assay to measure endogenous enzyme activity. In the case of 17β- HSD10, the enzymatic reaction rate increases with increasing substrate concentrations until a maximal velocity is reached as shown in figure 28.

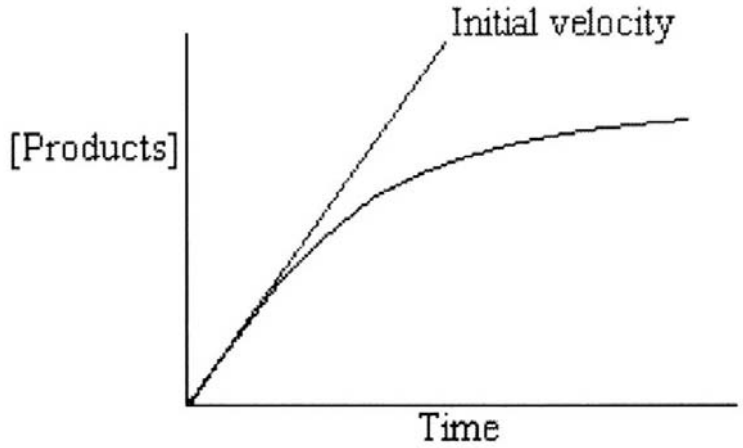


Figure 28 Time course of a single substrate enzyme reaction.

If a constant amount of enzyme reacts with varying concentrations of substrate, the reaction rate is directly proportional to substrate concentration in the initial velocity phase (v_0) whereas maximal reaction rate (v_{max}) is achieved when the enzyme is fully saturated with substrate. This relationship can be described by Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

The substrate concentration which gives half v_{max} is called Michaelis constant (K_m). The equation accounts for the kinetic data given in figure 28. At very low substrate concentrations, where the substrate concentration [S] is small compared to K_m, the reaction velocity (V) rate is directly proportional to the substrate concentration. At high substrate concentrations, where [S] is much higher than K_m, the reaction velocity is maximal and independent of substrate concentration. The plot of single substrate enzyme reaction is shown in figure 29. The Michaelis constant, K_m, and the maximal rate, V_{max}, can be readily derived from rated of catalysis measured at different substrate concentrations.

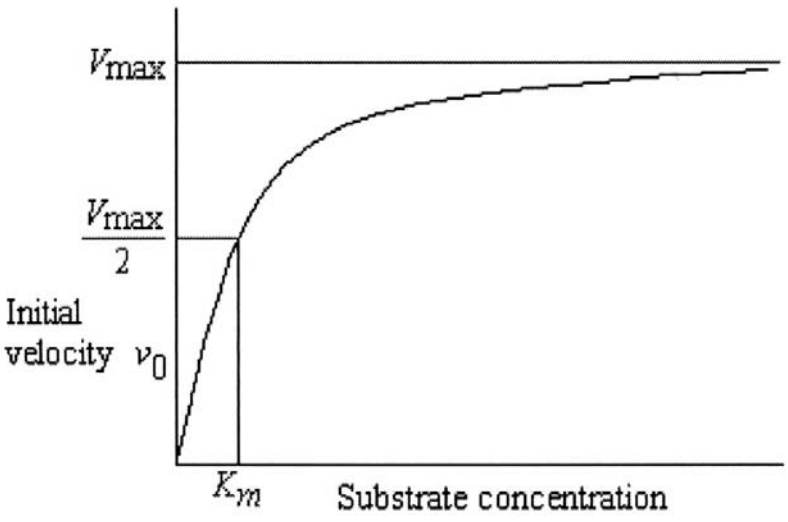


Figure 29. The dynamics of single substrate enzymatic reaction. Direct dependence of reaction velocity (v_0) on substrate concentration is observed when the enzyme is not fully saturated. Upon enzyme saturation, the reaction velocity reaches its maximum (V_{max}) and does not change in the presence of additional substrate. The substrate concentration which results in half of maximal reaction velocity is called Michaelis constant (K_m).

The kinetic characteristics of 17β- HSD10 as well as its substrate and coenzyme preferences were described by Yang et al. In this work, an assay for the assessment of enzymatic activity of endogenous 17βHSD10 was developed.

Due to unique -mitochondrial- localization of 17βHSD10, mitochondria which were extracted from rat hearts and mouse hearts were used as a source of 17β-HSD10. The analysis of 17β- HSD10 protein expression in mouse heart mitochondria showed comparable amounts of the enzyme in ERα- deficient and ERβ- deficient mice heart mitochondria comparing to their wild- type controls as shown in figure 30.

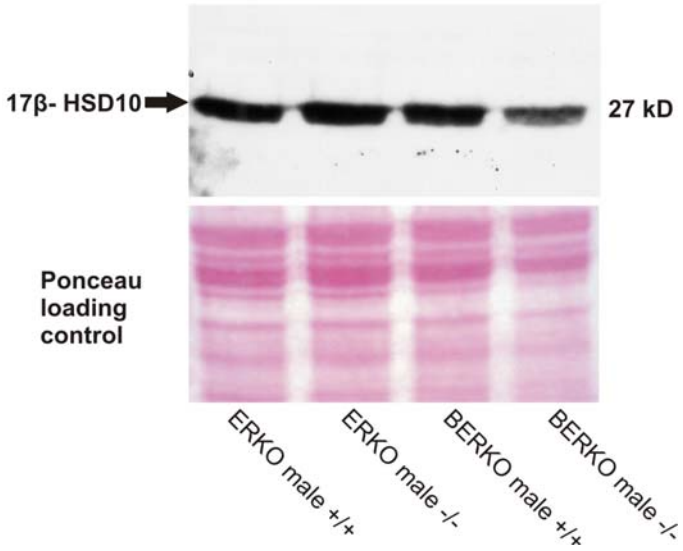


Figure 30. 17β-HSD10 protein expression in ERα (ERKO) and ERβ (BERKO) wild- type (+/+) and knockout (-/-) male mouse heart mitochondria. Western blot of 17β- HSD10 is shown in upper panel. Total protein staining of the same membrane using Ponceau reagent served as loading control.

The following parameters of the enzymatic assay were optimized in preliminary experiments:

1. The amount of mitochondria required for one reaction.
2. Optimal NAD^+ concentration.
3. The range of substrate 17β- estradiol at which 17β- HSD10 exerts its maximal activity.
4. Quantification of generated NADH and estrone.

100 µg of mitochondrial protein per assay generated measurable OD changes. Coenzyme NAD^+ titration experiments showed that maximal reaction efficiency was achieved using 200 µM NAD^+, whereas smaller amounts inhibited the reaction and higher concentrations did not influence the reaction velocity. The concentration of 17β- estradiol ranged from nanomolar to milimolar values. Continuous spectrophotometrical recording of OD changes at 340 nm provided a measure of NADH generation during a 30 minutes time course. The OD values corresponding to known NADH concentrations were spectrophotometrically determined at 340 nm. The Beer- Lambert law defines the linear relationship between absorbance and concentration of an absorbing species and is written

as: A=ε* b* c, where ε is the wavelength- dependent molar absorptivity (also called extinction coefficient), b is the path length and c is the analyte concentration. At 340 nm, the extinction coefficient of NADH (ε) is 6.2x 10^3 $M^{-1}cm^{-1}$. This coefficient was used in the calculations. In contrast to NADH, the extinction coefficient of NAD at 340 nm wavelength is close to zero and the changes in OD recorded by the spectrophotometer reflect only NADH generation. The following parameters were controlled in this enzymatic reaction: the generation of NADH by mitochondria alone i.e. basal mitochondrial activity (mitochondria in reaction buffer) and the production of NADH by adding additional NAD^+ (mitochondria+ NAD^+). All reactions were performed at pH 8.0. The curves obtained during spectrophotometric measurements are shown in figure 31. OD values obtained during measurements of the sample containing mitochondria and NAD but no estrogen served as baseline activity of 17β-HSD10 and were subtracted from ODs of the reactions containing estrogen. The resulting curves represent the enzyme activity in the presence of its substrate- 17β- estradiol.

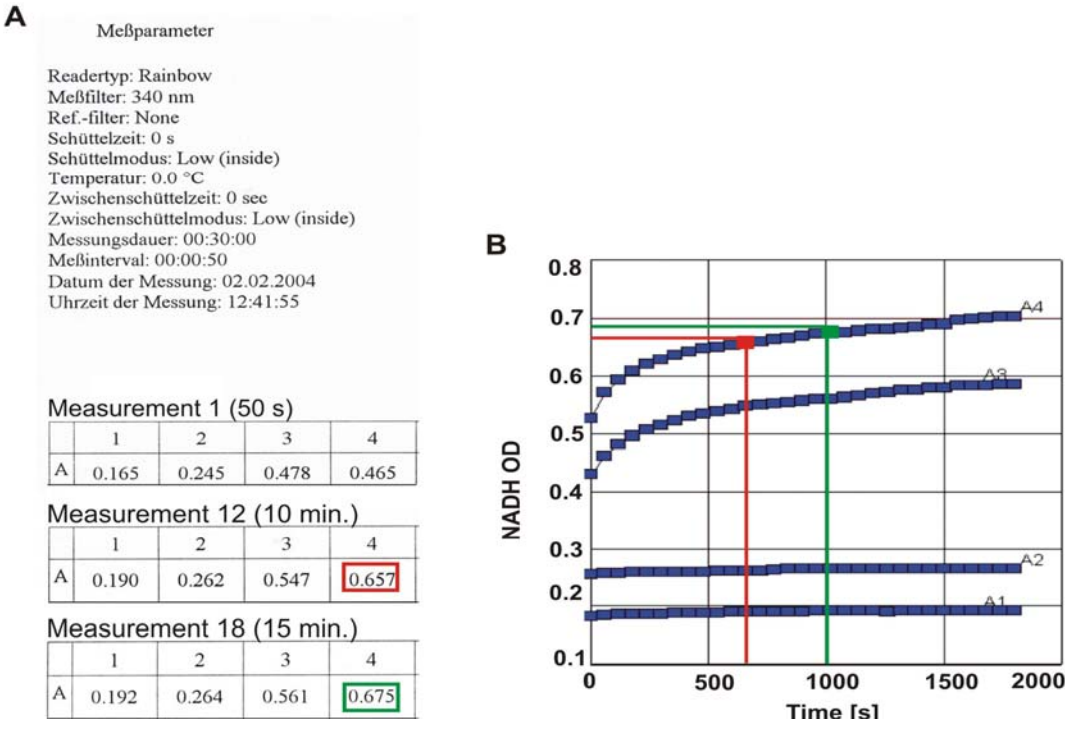


Figure 31. The curves obtained during continuous spectrophotometrical measurements of 17β-HSD10 enzymatic reaction. OD changes measured at 340 nm refer to the NADH changes during reaction. The table in panel A shows OD recorded at three time points (50s, 10 min. and 15 min.). Panel B represents the curves obtained during 30 minutes of reaction. A1 shows ODs generated by the enzyme alone whereas A2 refers to NADH generated by the enzyme in combination with exogenous NADH. The curves A3 and A4 represent NADH generation by endogenous 17β-HSD10, NAD and in the presence of two different substrate (estrogen) concentrations.

OD changes during 30 minutes reaction course is a measure for NADH generation. The absolute NADH values were estimated from a standard curve where known concentrations of NADH were spectrophotometrically measured at 340 nm. The results in figure 32 show the standard curve of NADH concentrations ranging from 10 µM to 500 µM.

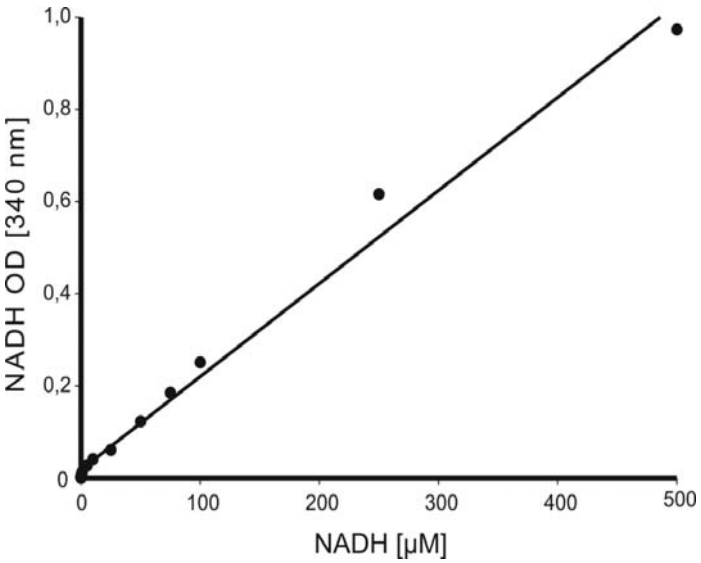


Figure 32. NADH standard curve. Pure NADH was dissolved in the reaction buffer used for 17β-HSD10 enzymatic assay (pH 8.0). The serial dilutions of NADH stock solution were performed and each sample was spectrophotometrically measured at 340 nm. The standard curve was created by plotting the absorbance for each standard on a linear y- axis against the concentration on the x-axis. The best fit curve was drawn through the points on the graph.

Absolute amounts of generated estrone were estimated using an ELISA assay kit. Enzymatic reaction samples, containing enzyme, generated NADH and estrone as well as negative controls were prepared for estrone measurements according to the manufacturer's protocol. Estrone was extracted from the reaction samples with methanol and immediately measured. The OD_{340} values of continuous recording using a microplate reader [wave length 340 mn] as well as estrone concentrations obtained from ELISA measurements are shown in figure 33.

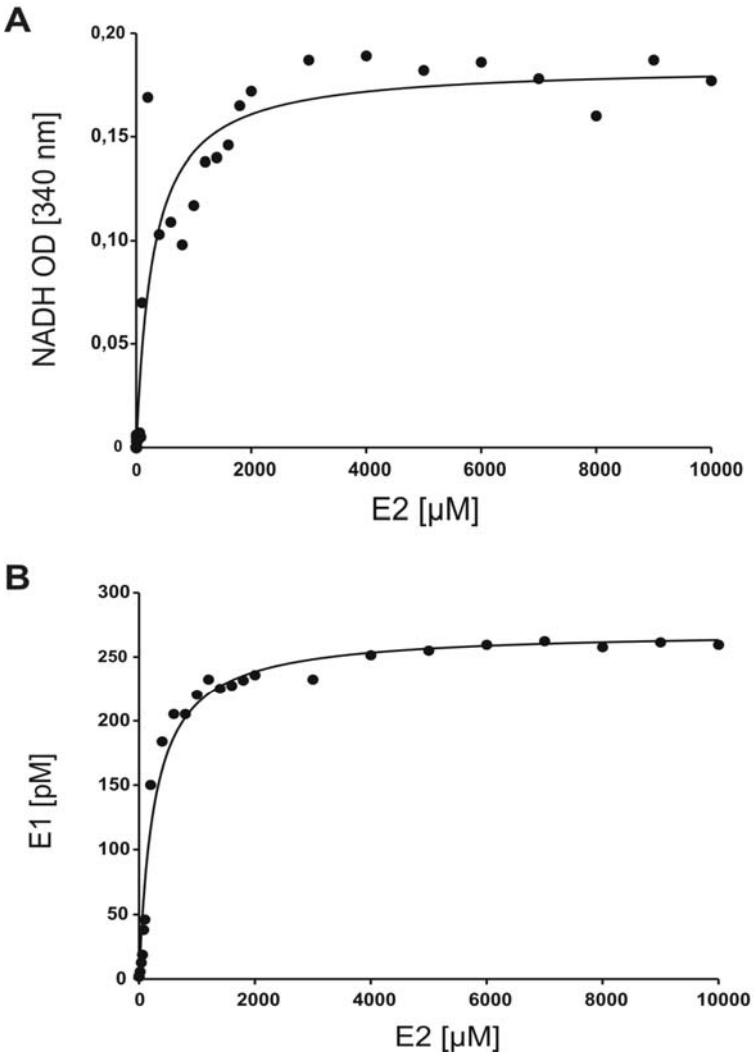


Figure 33. The dynamics of 17β- HSD10 and estrone generated during enzymatic reaction. The reaction graphic in panel A shows the ODs of NADH (Y axis) generated by adding ascending concentrations of 17β- estradiol (X axis). The absolute amounts of estrone (Y axis) generated during this reaction are shown in panel B. Estrogen concentrations used in this experiment are presented on X axis.

4.11. 17β- HSD10 activity studies in estrogen receptor deficient mouse heart

The influence of estrogen receptors alpha and beta on 17β- HSD10 activity was studied using estrogen receptor knock-out mouse models. Male mice were chosen for the experiments since male mice have relatively low serum levels of female sex hormones that could mask estrogen effects, and, therefore, impair the spectrophotometric measurements. In contrast to ER protein expression in rat hearts, neither ERα, nor ERβ protein could be detected in mouse heart. It could mean that the receptor expression levels are below the antibody detection levels. This hypothesis was supported by data obtained from RNA protection assays performed by Mendelsohn and colleagues. They could show ERα and ERβ estrogen receptor mRNA in wild- type mouse hearts and virtually no ERα and ERβ mRNA in ERα- and ERβ- knockout individuals, respectively (personal communication, data not shown). The ERα- deficient animals have been termed

ERKO and ERβ- deficient mice were designated as BERKO. Wild type animals are designated as “wt” whereas knockout animals are described as “ko”. These abbreviations will be used in the text.

The mitochondria from 3- 4 mouse hearts were pooled and used for one assay. Prior to enzymatic experiments, protein expression analyses of 17β- HSD10 and of both estrogen receptors were performed. As shown in figure 30, no significant differences could be detected in HSD expression in male ERKO and BERKO mouse as well as their wild- type control hearts. The 17β-HSD10 activity assays using mitochondria extracted from ERα- and ERβ- deficient mouse hearts were run in parallel with mitochondria obtained from wild- type littermate hearts. The data obtained from 7 independent experiments were evaluated and analysed using SigmaPlot 8.0 software. The Michaelis constant (K_m) derived from HSD curve analysis were comparable: K_m of 17β- HSD10 extracted from ERKO wt heart mitochondria was 301.4± 16 vs. 386.1± 30 μM in ERKO ko mice. K_m of BERKO wt animals was 445.7± 26 vs. 357.4± 26 μM in BERKO ko animals. These results show no significant differences in K_m of 17β-HSD10 extracted from wt mouse heart mitochondria. The results of the measurements are summarized in figure 34. Each point represents a mean of seven OD values obtained during seven independent experiments and the error bars are standard error of the mean. The best fitting curves were drawn through the points of the graph.

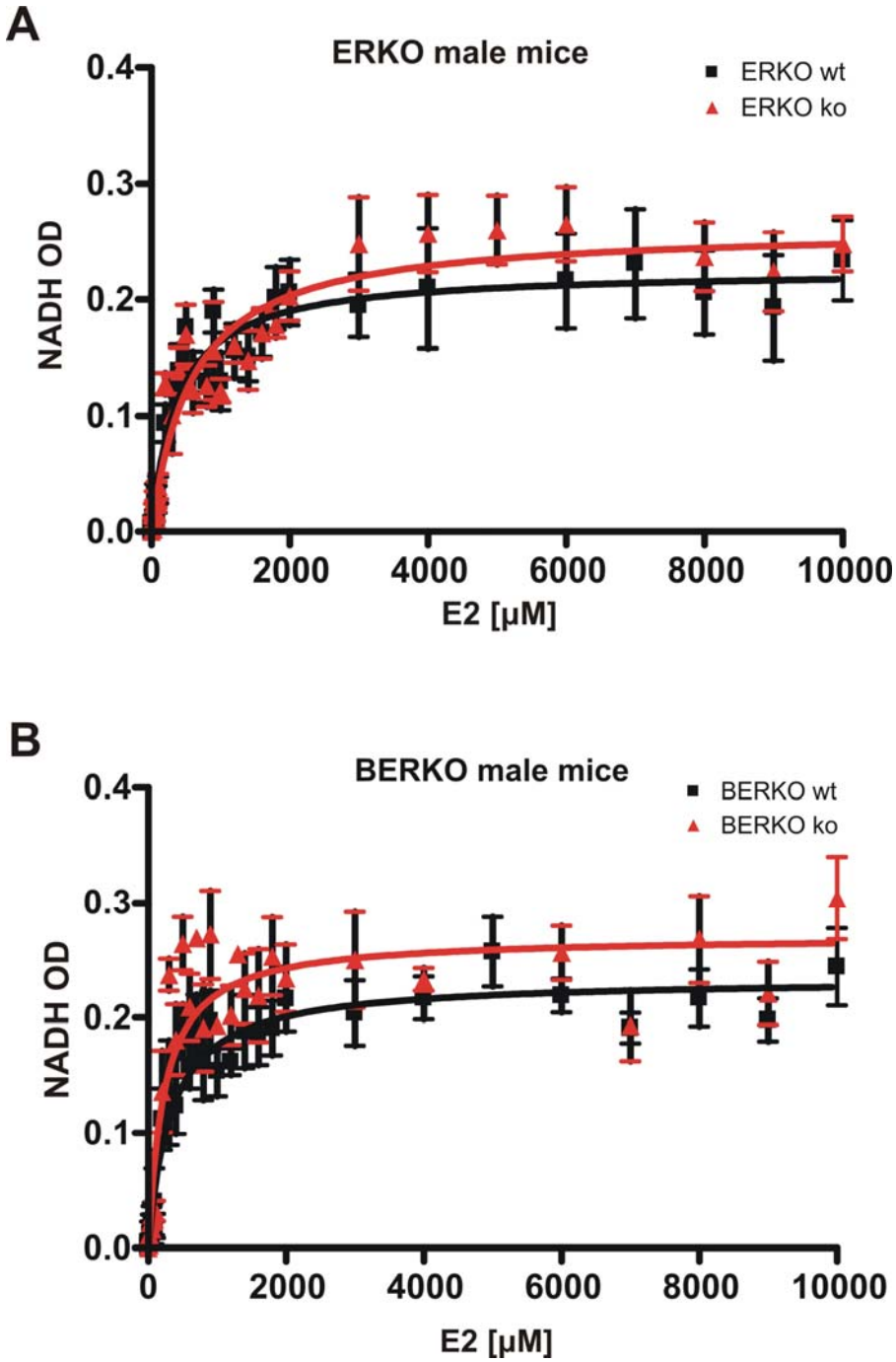


Figure 34. Summary charts of 17βHSD10 dehydrogenase assay using ERα- and ERβ- deficient mouse heart mitochondria. Each curve represents the average of seven independent experiments. The red curves show the results of enzymatic assay using mitochondria extracted from knockout mouse hearts whereas the black curves show the results of the experiments with wild- type mice.

4.12. Identification and characterization of BLCAP 10 in the heart

Bladder cancer- associated protein 10 (BLCAP10) was identified as protein-protein interaction partner of rERα_LBD in the embryonic human heart cDNA library. This 10 kD protein contains LXXLL motives that defines protein- protein interaction interface in estrogen receptors alpha and beta. The images of

BLCAP10 immunostaining in rat neonatal cardiac myocytes shown in figure 35 demonstrate BLCAP10 co-localization with ERβ rather than with ERα. Since both estrogen receptors possess two LXXLL motifs, it can not be excluded that BLCAP10 can interact with both estrogen receptors.

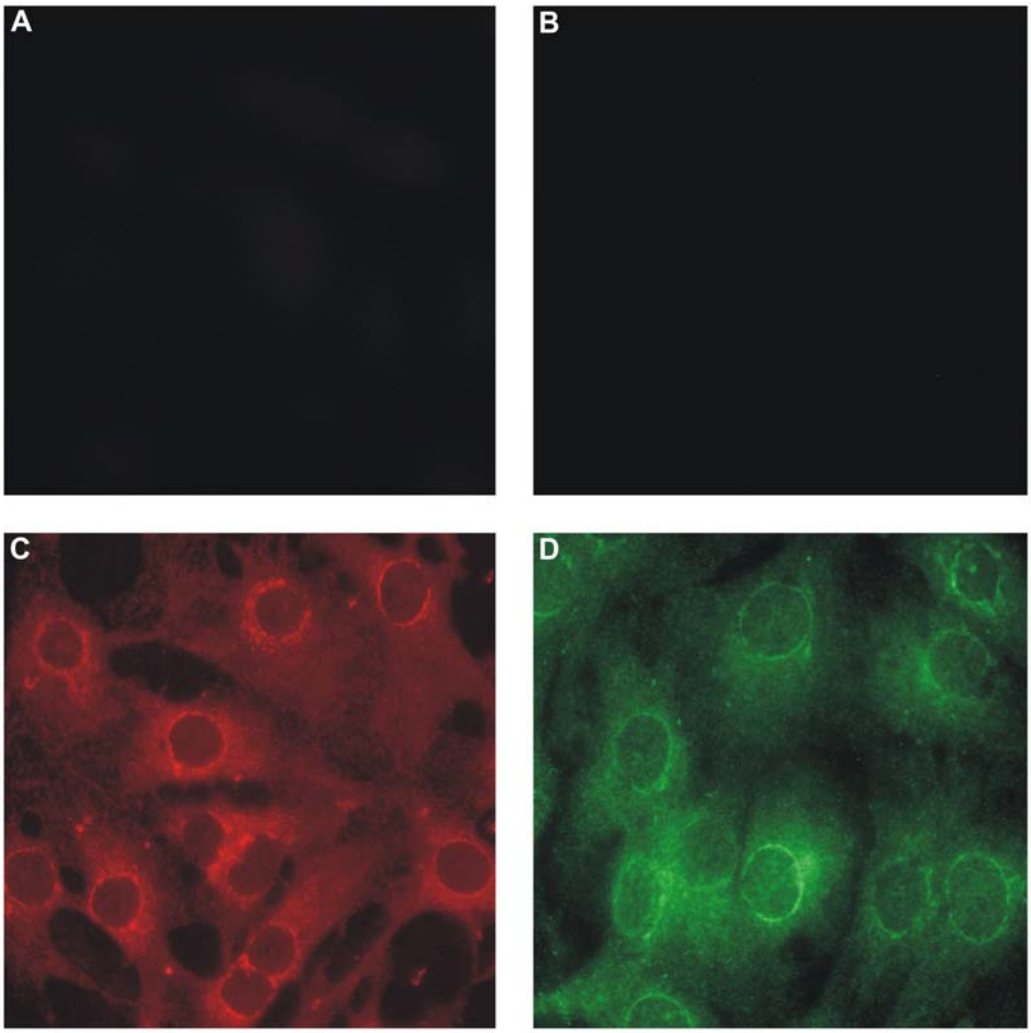


Figure 35. BLCAP10 immunostaining in rat neonatal cardiac myocytes. Cellular localization of bladder cancer associated protein 10 (BLCAP10) shown in panel D and ERβ (panel C) demonstrate similar- perinuclear- localization pattern in rat neonatal cardiac myocytes. Panels A and B show negative controls using goat anti- mouse conjugated with AlexaFluor 594 (red) or goat anti- rabbit conjugated with AlexaFluor 488 (green) fluorescent dye.

The screening of human BLCAP10 protein sequence (PubMed acc. Nr. NP_006689) for transmembrane segments using DAS software (Stockholm Bioinformatics Centre) revealed two potential transmembrane domains. The first domain was localized within amino acids 370- 380 and the second one spanned the region between amino acids 575- 587. BLCAP10 also contains two LXXLL domains which correspond to amino acids 355-359 and 372-376. Since the latter

LXXLL spans the potential transmembrane domain, one could speculate that this LXXLL is “buried” in the membrane and, therefore, cannot interact with the proteins. The transmembrane segment prediction charts as well as protein sequence with indicated LXXLL motifs are shown in figure 36.

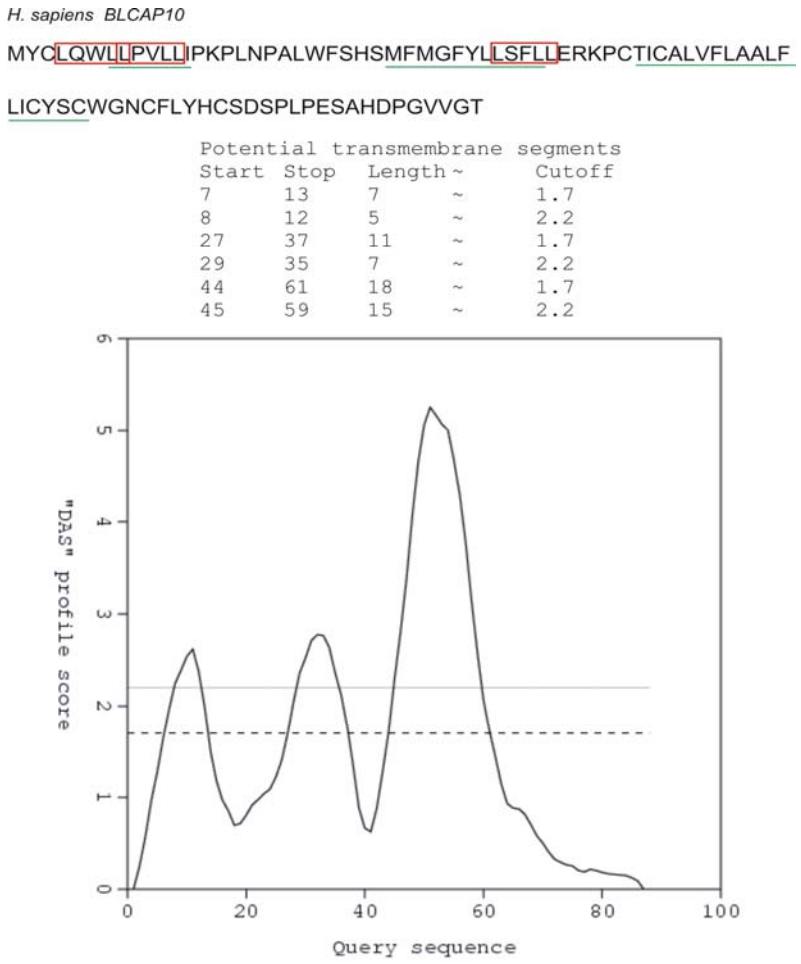
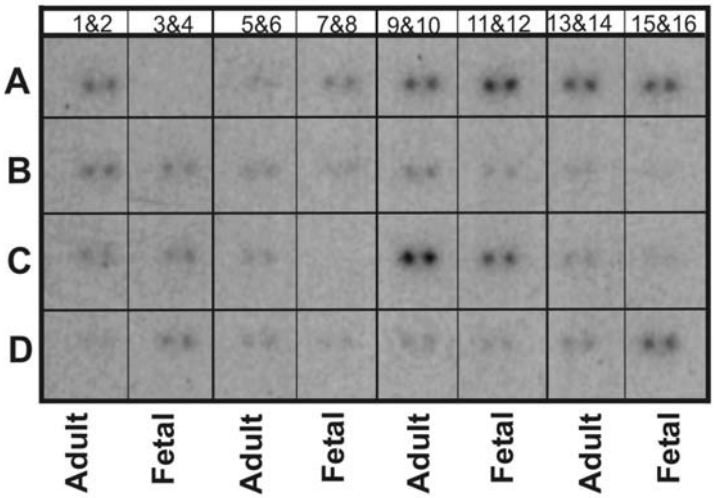


Figure 36. Potential transmembrane segments of human bladder cancer- associated protein 10 (BLCAP10). BLCAP10 protein sequence is shown in upper panel where protein- protein interaction motifs LXXLL (X- any amino acid) are shown in red frames whereas potential transmembrane segments are highlighted in green. The graphical representation of the sequence analysis is shown in lower panel. The curve was obtained by pairwise comparison of the proteins in the test set in "each against the rest" fashion. There are two cut-offs indicated on the plot: a "strict" one at 2.2 DAS score, and a "loose" one at 1.7. The hit at 2.2 is informative in terms of the number of matching segments, while a hit at 1.7 gives the actual location of the transmembrane segment.

The protein expression analysis of BLCAP10 in the heart using western blotting failed because of technical problems. So far, BLCAP10 mRNA and protein expression were detected in non- invasive human bladder carcinoma cell lines[141]. Therefore, the screening for BLCAP10 mRNA was performed using human tissue mRNA array purchased from Biocat. Surprisingly, BLCAP10 mRNA was moderately expressed in fetal and adult bladder (see figure 37, sections A 5&6, A

7&8). Minimal amounts of mRNA could be detected in adult heart (B 5&6) and minor expression levels were detected in fetal heart (B 7&8). The highest mRNA levels were measured in fetal and adult brain (A 9&10, A 11&12), cerebellum (A 13&14, A 15&16), skeletal muscle (C 9&10, C 11&12) and fetal tongue (D 15&16). The array data shows that BLCAP10 message is expressed predominantly in the tissues that consist of terminally differentiated cells, i.e. neurons (brain and cerebellum) and myocytes of skeletal muscle and tongue but at lower levels also in cardiomyocytes.

A



B

Array Diagram:

	1 & 2	3 & 4	5 & 6	7 & 8	9 & 10	11 & 12	13 & 14	15 & 16
A	Positive Control	Negative Control	Adult Bladder	Fetal Bladder	Adult Brain	Fetal Brain	Adult Cerebellum	Fetal Cerebellum
B	Adult Colon	Fetal Colon	Adult Heart	Fetal Heart	Adult Kidney	Fetal Kidney	Adult Liver	Fetal Liver
C	Adult Lung	Fetal Lung	Adult Skin	Fetal Skin	Adult Skeletal Muscle	Fetal Skeletal Muscle	Adult Small Intestine	Fetal Small Intestine
D	Adult Spleen	Fetal Spleen	Adult Stomach	Fetal Stomach	Adult Thymus	Fetal Thymus	Adult Tongue	Fetal Tongue

Figure 37. BLCAP10 gene expression levels in adult and fetal human tissue mRNA array. Human mRNA array was screened using radioactive human BLCAP10 cDNA probe. The results are shown in the radiogram (A). The fist two pairs of spots in row A are positive and negative controls respectively. Spot identities are listed in Array Diagram (B).

5. Discussion

Cardioprotective effects of estrogens have been observed long before the discovery of functional estrogen receptors in male and female hearts [15]. The presence of functional ERα and ERβ in the heart led to the hypothesis that the heart might be a target organ for estrogens. Multiple observational studies showed a lower incidence of cardiovascular disease in premenopausal women compared to their age- matched male counterparts [3]. Therefore, estrogen effects in the heart were extensively studied in animal models [142-144] as well as in multiple epidemiological studies [145, 146]. Experimental studies showed that physiological dosages of 17β- estradiol effectively attenuated cardiac hypertrophy and improved cardiac function in young hypertensive animals [115, 137]. In contrast, controlled clinical studies such as HERS [6] and WHI [147] showed that classical estrogen- progesterone replacement therapy in post- menopausal women did not reduce the overall rates of cardiovascular events. Moreover, increased rate of negative side effects was observed and estrogen itself increased the risk of endometrial and breast cancer. Due to clinical relevance and due to scientific interest, synthesis of novel substances which selectively bind to either ERα or ERβ gained considerable attention during past several years. J. Katzenellenbogen and colleagues developed several synthetic estrogen receptor agonists which selectively bind to either ERα or ERβ. Another approach in generating ER- isoform selective ligands was applied by Hillisch et al [111]. They studied structural differences of the ligand binding pocket of both estrogen receptors and modified the three- dimentional structure of 17β- estradiol in a way that an ERα agonist 16α-LE_2 could enter ligand binding pocket of ERα but not of ERβ. The same principle was applied for the synthesis of 8β- VE_2, a selective ERβ agonist. The first *in vivo* studies using both agonists showed differential effects of each ER isoform in the mouse reproductive system [108]. In the current project, *in vivo* evaluation of 16α-LE_2 and 8β- VE_2 on the cardiovascular system of female SHR rats was performed. The majority of pre-clinical studies have been performed using young, “pre-menopausal” animals. In the clinical practice, however, estrogen substitution is applied to elderly, post- menopausal women[148, 149]. In the current study, physiological effects of 16α-LE_2 and 8β- VE_2 in young “premenopausal” and senescent “postmenopausal” female SHR rats were

compared with that of 17β- estradiol. This study was designed to answer the following questions: 1) is estrogen substitution therapy in senescent animals as effective as in young female SHRs and 2) does selective activation of endogenous ERα or ERβ results in differential effects on cardiovascular system? Special attention was paid to cardiac function and gene expression, in particular to contractile proteins and to calcium- transporters. Estrogen receptors can be regulated not only by ligands but also by other proteins such as cofactors via direct protein- protein interaction. Therefore, a study on detection and characterization of novel ERα protein- protein interaction partners was initiated. Two possible ERα interaction partners, 17β- hydroxysteroid dehydrogenase type 10 (17β-HSD10) and bladder cancer associated protein 10 (BLCAP10) were characterized in more detail.

5.1. Influence of hormone replacement on morphological parameters

Body weight together with uterus weight served as global parameters for determination of substitution efficacy for young and senescent SHRs with estrogen and estrogen receptor selective ligands. Body weight was comparable between young and senescent intact female spontaneously hypertensive (SHR) rats. Ovariectomy resulted in significant increase of body weight in both, young and senescent rats. Body weight was effectively reduced in 17β- estradiol and selective ERα agonist 16α-LE_2- treated young and senescent ovx. The role of ERα and ERβ in body weight control was extensively studied in ERα and ERβ-deficient mice. Heine and collegues could show that the lack of ERα in female mice resulted in significant accumulation of white adipose tissue, significant increase in fat pad weight, adipocyte size, and number compared to sham operated ER- knockout mice [150]. In contrast, removal of E2/ERβ signaling in αERKO mice by ovariectomy decreased body and fat-pad weights and adipocyte size. ERβ mediated effects on adipose tissue are opposite to those of ERα, although E2 effects on adipose tissue are predominately through ERα. In addition, ovariectomized ERα knockouts showed a trend towards decreased food consumption that did not reach significance [151]. Therefore, the observation that selective activation of ERβ by 8β-VE_2 did not influenced body weight of young ovx rats is in agreement with the data obtained from the studies on ERβ-deficient mice [152]. Surprisingly, treatment with 8β-VE_2 resulted in significant decrease of body weight in senescent ovariectomized SHR rats. In view of

unexpected results, additional studies would be necessary to confirm this observation. Uterus highly expresses ERα and is a "classical" target organ for estrogen. Therefore, uterus weight is another well established physiological criterion for determination of estrogen or estrogen- like compound efficacy under experimental conditions [153, 154]. The physiological role of both estrogen receptor isoforms were studied using ERα and ERβ knockout mouse models. Targeted disruption of ERα in female mice caused infertility and uterus atrophy that is refractory to estrogens. In contrast, ERβ knockout females exhibited a hormonally responsive uterus and grossly normal ovaries.[155]. In the current study, absolute uterus weight measurements showed that physiological amounts of estrogen and pharmacological dosages of 16α-LE_2 effectively increased uterus weight in both age groups. In contrast, selective ERβ activation by 8β-VE_2 did not influence this parameter, i.e. the uterus weight was comparable with the uterus weight of ovariectomized rats. These findings clearly show that both, 16α-LE_2 and 8β-VE_2, are ERα and ERβ selective ligands and do not cross- react with the other ER isoform *in vivo*.

Cardiac hypertrophy is frequently associated with hypertension and is an independent predictor of cardiac mortality [156]. The degree of cardiac hypertrophy was assessed by normalizing absolute heart weight versus tibia length. The ovariectomy of young SHR females resulted in increased cardiac hypertrophy which could be attenuated by estrogen treatment. This observation is in agreement with the data reported by our group and others [14, 87, 157]. Substitution of young ovariectomized SHR rats with 16α-LE2 and 8β-VE_2 effectively attenuated cardiac hypertrophy in young ovx. These results showed that selective ERα activation reduced cardiac hypertophy directly and without influencing blood pressure whereas ERβ agonist effectively reduced cardiac hypertrophy via reduced blood pressure. The results of myosin heavy chain expression analysis as well as hemodynamic data will be discussed in the sections 6.4 and 6.2, respectively

In contrast to young SHRs, ovariectomy and subsequent treatment with estrogen or ERα and ERβ agonists did not influenced cardiac mass in senescent SHRs. This observation is in agreement with the data published by Sharkey and co-workers who have investigated the effects of estrogen substitution on the heart of senescent, heart- failure- prone (hfp)- rats (Heart- Failure-Prone SHHF/Mcc-fa^{cp}) [158]. They showed that absolute heart weight of 17-months old hfp- rats was not

affected by estrogen treatment. In their study, plasma estrogen levels were measured only in young but not in senescent rats. The lack of complete hemodynamic data, estrogen plasma level measurements in senescent hfp- rats as well as ER protein expression analysis in the hearts both age groups did not allow direct comparison of estrogen treatment efficiency in young and senescent hfp- rats.

In this study, complex approach for assesment of cardioprotective effects of estrogens was applied which included not only morphometric data and assessment of cardiac hypertrophy but also cardiac function analysis using invasive hemodynamic technique.

5.2. Influence of estrogens on cardiac function

Data obtained during invasive hemodynamic analysis of both SHR age groups allowed direct comparison of cardiac function in young and senescent animals. The influence of estrogen, ERα agonist 16α-LE_2 and ERβ agonist 8β-VE_2 on cardiac function was analysed in both age groups. Elevated blood pressure was observed in both, young and senescent SHR animals. Selective activation of ERα did not reduce elevated blood pressure in young ovx rats. In contrast, left ventricular pressure and aortic pressure in young animals was significantly lower in ovx rats substituted with ERβ agonist 8β-VE_2. Another important functional parameter is cardiac output which is expressed as the volume of blood being pumped by the heart per time unit. Increased cardiac output was observed in young rats that were ovariectomized and substituted with either ERα or ERβ agonist. Both agonists also were effective in reducing cardiac hypertrophy. Estrogen, however, effectively reduced cardiac mass but only slightly increased cardiac output in young ovariectomized rats. The experimental data about estrogen influence on hemodynamic parameters obtained in our laboratory are in agreement with the observations published by other research groups [87, 158]. Hemodynamic data suggested different regulatory function of estrogen receptors: ERα could improve cardiac function while reducing cardiac hypertrophy whereas ERβ significantly increased cardiac output by reducing blood pressure *and* cardiac hypertrophy. A possible explanation for this phenomenon could be differential estrogen receptor isoform distribution in the cardiovascular system. This hypothesis was supported by the data obtained during ER protein expression analyses in the cardiovascular system of SHR rats. We could show

that ERα is more abundant in the heart whereas ERβ was the dominant isoform in the aorta. In senescent rats, estrogen and both ER agonists did not significantly influence hemodynamic parameters, i.e. left ventricular systolic pressure was notably higher compared to young animals whereas heart rate was substantially decreased. Substitution of senescent ovx rats with either 16α-LE_2 or 8β-VE_2 did not lowered blood pressure. The fact that senescent cardiovascular system did not responded to the treatment raised the following questions: 1) are both ER isoforms expressed in the senescent heart, 2) if yes, can ERs still respond to the ligand treatment, or 3) did advanced hypertrophy cause "cardiac "stiffness" with subsequent worsening of cardiac function in senescent animals? The data of estrogen receptor protein expression analysis showed that both ER isoforms are expressed in senescent myocardium. Moreover, expression levels of both receptors could be regulated by estrogen, 16α-LE_2 and 8β-VE_2. Protein expression data will be discussed in the section "Protein expression analysis". Additional studies such as determination of collagen content in the senescent heart could help to define the degree of cardiac stiffness.

Estrogen and, in particular, estrogen receptor selective ligands were efficient cardioprotective substances in young rats under experimental conditions where they could reverse cardiac hypertrophy and/or improve cardiac function. The substances, however, were not effective in senescent animals. Therefore, the age of the laboratory animals plays a crucial role in successful outcome of estrogen and estrogen- like substance treatment.

5.3. Sex hormone profiles

To evaluate cardiovascular estrogen effects in young and senescent rats requires knowledge about endogenous sex hormone levels (estrogens and androgens). Thus, the following hormones were measured in plasma samples: estrogen (E2), two estrogen precursors- testosterone (T) and 4- androstenedione as well as estrogen metabolites estrone (E1) and estriol (E3). The major and surprising finding of this study was different estradiol metabolism in senescent rats compared to young ones. As already mentioned, the blood samples in all treatment groups were collected between 14 and 17 hours after last estrogen injection. Hormone measurements were performed the same day. Plasma estrogen levels in young ovariectomized SHR rats were significantly lower compared to sham operated animals. Estrogen injections resulted in full recovery

of plasma estrogen levels in young rats. These data, together with uterus weight provide valuable information not only about estrogen substitution efficacy but also about the quality of ovariectomy. Surprising results were obtained after the measurements of 17β- estradiol content in the plasma of senescent SHR rats. According to the literature, rats undergo menopause at the age of 10 to 12 months [120] with gradually declining estrogen levels. Low estrogen levels in both, sham and ovariectomized, senescent rats were in agreement with published data[158]. The finding that estrogen levels in estradiol substituted senescent rats were as low as in ovx rats receiving placebo was unexpected. Effective regulation of physiological parameters such as body weight and uterus weight together with estrogen receptor protein expression data showed that senescent rats were sufficiently substituted with estrogen. Blood sample collection from young and senescent animals was performed within identical time interval after hormone injections. Discrepancy between physiological parameters and low estrogen levels in substituted rats suggested that probably different estrogen metabolism in young and senescent rats could play a role. Therefore, two major estrogen metabolites- estrone and estriol- were measured in all plasma samples. As expected, plasma estrone levels in young rats were high in sham operated and estradiol substituted rats, whereas significant decrease of this hormone was observed in ovariectomized individuals and in the rats treated with ER selective ligands. In senescent rats, estrone levels were lower compared to young animals and were comparable among the treatment groups. In contrast to estrogen and estrone, plasma estriol levels in young SHR rats were similar in all treatment groups. Little is known about circulating estriol levels in post- menopausal individuals, therefore estriol plasma levels were estimated in the current project. The measurements showed almost two times higher estriol levels in sham operated senescent rats compared to their young counterparts. Low E3 serum concentrations could be detected in senescent ovx and ovx substituted with estradiol and both agonists. Major estrogen precursor testosterone levels were high in young sham operated rats and low in ovariectomized ones. Estrogen synthesis from testosterone is irreversible process. Therefore, estrogen injections could not influence testosterone levels. Lower, but still measurable testosterone concentrations could be observed in plasma of senescent rats. As in the case of young SHRs, ovariectomy resulted in massive decrease of testosterone in the plasma of senescent rats. These results strongly suggest that in the aged

individuals, estrogen metabolism but not synthesis play a pivotal role in hormone homeostasis. It is likely that exogenous estrogen could not exert its cardioprotective properties due to its short half-life in senescent rats compared to young ones. Studies of the enzymes which are involved in estrogen synthesis (aromatase) and metabolism (hydroxysteroid dehydrogenase isoforms 2, 4, and 8) and their kinetic properties in both, young and senescent individuals could help to understand better the results of hormone measurements performed in this project.

5.4. Cardiac myosin heavy chain expression

Myosin heavy chain is major contractile protein in the heart. Under physiological conditions, myosin together with other myofibrillar proteins such as actin, tropomyosin and troponin forms functional myofibrils. Two myosin heavy chain isoforms- alpha and beta (α- and β- MHC) are ATP- dependent cardiac "motors" responsible for cardiac contraction. Myosin consisting of αMHC has higher ATPase activity than myosin composed of βMHC. Thus, hearts expressing αMHC have more rapid contractile velocity than hearts expressing βMHC, which allows greater economy in force generation [159]. The ratio between α- and β- MHC in the heart is species specific: in healthy mouse heart, αMHC is the dominant isoform whereas βMHC dominates in healthy human heart. In the rat heart, both, α- and β-MHC are expressed although αMHC prevails over βMHC. In hypertrophied rat heart, α- to β- MHC ratio is shifted toward βMHC [160]. This α- to β isomyosin "switch" is characteristic adaptation because it lowers wall stress of hypertrophied heart [161-163]. Cardiac hypertrophy is also characterized by an increase in cell size in the absence of cell division and is accompanied by a number of qualitative and quantitative changes in gene expression. On the level of gene expression, upregulation of the so-called fetal genes, i.e., βMHC, alpha-skeletal and alpha-smooth muscle actin, and atrial natriuretic factor (ANF) may be observed concomitant with a downregulation of αMHC and the Ca^{2+} pump of the sarcoplasmic reticulum (SERCA2a) [164]. Decreased αMHC expression or mutations in αMHC gene can lead to dilation and systolic dysfunction in the human heart [165] whereas increased βMHC expression in rat myocardial preparations led to significant depression of contractile function in thyroid deficient myocardium by slowing the rates of both force development and force relaxation. In the current study, protein expression levels of αMHC were higher

compared to βMHC in sham operated young animals whereas α- to- β- MHC ratio has been "switched" towards βMHC in ovariectomized young rats. The substitution of ovariectomized young rats with estrogen and 16α-LE_2 induced αMHC protein expression and the α/β MHC ratio was comparable with that of sham operated individuals. Selective activation of ERβ by 8β-VE_2 did reduce heart weight compared to the ovariectomized group but did not affect MHC expression. These results indicate that estrogen reduces cardiac hypertrophy in rat heart by upregulation of αMHC protein synthesis via ERα, but not via ERβ. Gupta and colleagues reported that serum- inducible immediate early gene protein Egr-1 regulates the transcription of αMHC gene[79]. It was reported that Egr-1 can be upregulated by estrogen via extracellular signal-regulated kinase, ERK1/2 and serum response elements (SREs) but not via the estrogen response elements or AP-1 sites [42]. Further the αMHC promoter analyses are required to determine whether ERα directly interacts with αMHC promoter or indirectly via other transcription factors that bind to the αMHC promoter. Despite sufficient substitution with estrogen and ER selective ligands neither ovariectomy, nor substitution with estrogen, 16α-LE_2, or 8β-VE_2 influenced the heart weigh of senescent individuals. These results suggest that 1) senescent hearts did not respond to physiological dosages as effectively as young ones and 2) estradiol and 16α-LE_2 substitution did not result in higher αMHC expression because of extremely low ERα levels of even absence of the receptor in senescent hearts. As already discussed in the previous chapter, estrogen metabolism in senescent rats was much higher compared to young rats. Estrogen receptor alpha protein expression showed low receptor levels in senescent hearts and its expression was restored by estrogen and ERα agonist. Although plasma estrogen levels remained low in E2 substituted senescent rats, ERα expression was upregulated by estrogen and 16α-LE_2. ERα and ERβ protein expression results are discussed in the following section.

5.5. Protein expression analysis

Biological function of estrogens is mediated by two different estrogen receptor subtypes, ERα and ERβ, which are expressed and functionally active in numerous tissues and cells, including cardiomycytes and vascular smooth cells. Estradiol is known to regulate ERα protein expression in other tissues and cell types such as endothelial cells[166], Ishikawa cells[167] and hippocampus[168].

Therefore, we postulated that ERα protein expression levels would be higher in estrogen or selective ligand substituted rat hearts. This hypothesis could not be confirmed by the data obtained from ERα expression analysis in the young rat hearts because ERα was expressed in comparable amounts and was not influenced by hormones. Estrogen receptor alpha protein expression analysis in senescent hearts, however, showed extremely low protein levels in sham and ovx rats whereas estradiol and 16α-LE_2 injections fully restored receptor expression which was no longer different from ERα expression in young rats. These data are in agreement with the data published by Ihionkhan and colleaques who showed that estrogen upregulated ERα protein expression in endothelial cells [166]. 8β-VE_2 did not influence ERα expression in senescent hearts and ERα protein levels were comparable with those of sham and ovx rats. Estrogen receptor beta protein expression in young animals was similar among the treatment groups. ERβ expression in senescent hearts was also comparable among the groups exept the animals substituted with 16α-LE_2. In this group, selective activation of ERα led to a downregulation of ERβ protein expression. Additionally, ERα expression upregulation with estrogen and ERα- selective agonist could serve as molecular marker for controlling substitution efficacy in the future experiments.

Protein expression analysis of calcium handling proteins, such as SERCA2, phospholamban and its phosphorylated form phospho-phospholamban showed only minor differences in protein expression and phosphorylation levels between young and senescent SHR rats or among the treatment groups. These data are in agreement with published results that showed no significant differences in SERCA, phospholamban (PLB) and phosphorylated phospholamban (pPLB) protein expression in the hearts of ovariectomized and estrogen or estradiol substituted rat [169, 170]. Protein expression of another calcium handling protein ryanodine receptor (RyR) was shown to be unaffected in the rats treated with estradiol benozoate for two weeks [169] but upregulated after six- week estrogen injections [171, 172].

Protein expression analysis in the senescent rats showed that estrogen and both estrogen receptor ligands only moderately influenced the expression of calcium handling proteins in the heart. A non- significant decrease in SERCA protein expression levels was observed in senescent hearts compared to young hearts. The reduction of SERCA2 gene expression has been observed also in aged

human heart. This age-associated reduction in transcription of the SERCA2 gene could be, at least, partially responsible for a decrease in the SR pump site density [172, 173]. As in young rats, estrogen and both estrogen receptor selective agonists did not influence SERCA2 expression in senescent ovariectomized rats. Protein expression analysis of phospholamban and its phosphorylated form showed a non- significant increase in PLB levels in ovariectomized and estrogen substituted senescent rat hearts compared to young rats. The major finding of this study was that estrogen could effectively reverse cardiac hypertrophy in young animals whereas in senescent rats, treatment with estrogen was not effective. Estrogen receptor isoform selective ligands were superior to estrogen in reducing cardiac hypertrophy and improving heart function of young SHR rats whereas in senescent rats the treatment resulted in non- significant effects on the heart. These observations suggest that treatment with estrogen and estrogen-like substances might be efficient if started soon after onset of the menopause in the rats, i.e. at the age of 10 to 12 months.

5.6. Protein- protein interaction partners of ERα in the heart

Human embryonic heart cDNA library screening for estrogen receptor alpha protein- protein interaction partners identified several potential interaction partners for ERα, which have so far not been described. The ERα binding domain was used as a bait in the cDNA library screening experiments. The most striking finding of this project was that almost 23 percent of the obtained positive clones contained mitochondrial genes (28 clones out of total 120 clones). The cellular localization pattern of ERα in neonatal rat cardiomyocytes showed that the receptor is present not only in the nucleus but also in cytosol. This cytosolic fraction had characteristic pattern which matched partially with that of mitochondria. Co- localization experiments confirmed that a fraction of ERα is localized in mitochondria. As already discussed in the *Results*, ERα specific antibody used for co-localization studies was tested for its specificity using wild-type and ERα- deficient mouse liver sections. These data partially agree with the observations made in MCF7 cells where both estrogen receptor isotypes could be shown in mitochondria using immunocytochemical and western blot techniques [68]. However, the majority of the published results have shown that ERβ but not ERα is localized in mitochondria of human lens epithelial cells, osteosarcoma and hepatocarcinoma cell lines [69, 174]. In contrast to the publihed

data, estrogen receptor beta immunostaning performed in the current study showed that ERβ was localized mainly perinuclearly and could not be detected in mitochondria of neonatal cardiac myocytes.

17β- hydroxysteroid dehydrogenase type 10 is one of the most interesting potential protein- protein interaction partners for ERα identified in the neonatal human heart cDNA library. This protein was identified and characterized by Yang and colleagues in 1998 [136]. This enzyme was detected in human brain cDNA library screening experiments and assigned as Short Chain L-3- hydroxyacyl-CoA Dehydrogenase (SCHAD). This fatty acid β- oxidation enzyme was identified as a novel 17β- hydroxysteroid dehydrogenase responsible for the inactivation of sex steroid hormones and was assigned as 17β- hydroxysteroid dehydrogenase type 10 (17β-HSD10) [139]. In the current project, we showed for the first time that ERα co-localizes and directly interacts with 17β- HSD10 under estrogen- free conditions. Addition of estrogen blocks this interaction. Since 17β-HSD10 lacks LXXLL motif which is frequently involved in estrogen receptor interaction with other proteins, one would expect that ERα- 17β-HSD10 interaction, if it takes place, would be transient. This hypothesis was confirmed by the data using HELA cells co-expressing recombinant 17β-HSD10 and ERαLBD. If HELA were cultivated in the medium containing foetal calf serum, co-immunoprecipitation failed whereas elimination of estrogen from cell growth medium using charcoal- stripped serum resulted in positive co-immunoprecipitation. These results suggested that this transient interaction takes place only in the absence or in the presence of minor amounts of estrogen. Since 17β-HSD10 metabolizes and thereby inactivates estrogen, it could be possible that in the case of low E2 levels, ERα interacts with HSD and temporary inhibits estrogen conversion to estrone. In the presence of high estrogen amounts, 17β-HSD10- ERα might dissociate and the dehydrogenase would exert its maximal activity. We also showed for the first time the dehydrogenase activity of endogenous 17β-HSD10. Due to its mitochondrial localization, enriched mitochondrial fraction could be used as a source of endogenous 17β-HSD10 protein and its function could be analyzed spectrophotometrically. Reactions were performed under alkalic conditions (pH 8.0) in the presence of endogenous enzyme, coenzyme NAD^+ and various concentrations of 17β- estradiol. Protein expression analysis revealed that 1) 17β-HSD10 was expressed in comparable amounts in ERα and ERβ knockout and wild- type control male and female

mouse hearts and liver and 2) expression of both ER isoforms in mouse but not in rat hearts was below antibody detection level. Therefore, we hypothesised that estrogen receptor- associated effects on HSD function might be minimal in mice. The data obtained during enzymatic assays show slightly higher enzyme activity in the reaction where either ERα or ERβ was missing. Mouse liver express detectable amounts of estrogen receptor, therefore liver mitochondria of ERα and ERβ null- mice were used as alternative 17β- HSD10 source. In contrast to successful experiments with heart mitochondria, spectrophotometric measurements of 17β- HSD10 enzymatic activity failed when liver mitochondria were used. Abundance of highly active dehydrogenases that metabolise estradiol to estrone, such as 17β- hydroxysteroid dehydrogenases 2, 4 in the liver [175] might be responsible for the failed experiments.

The second ERα protein- protein interaction partner obtained from the human heart cDNA library was identified as bladder cancer associate protein 10 (BLCAP10). This 10 kD protein was detected in the non-invasive bladder cancer lesions and downregulated in the invasive human transitional cell carcinomas[141]. To date, there are no data available about the cellular localization and tissue distribution of BLCAP10. Immunostaining of the neonatal cardiac myocytes with rabbit polyclonal anti- BLCAP10 (kindly provided by Dr. I. Gromova from the Institute of Cancer Biology in Kopenhagen, Denmark) resulted in strong perinuclear staining which was characteristic for ERβ but not for ERα. The mRNA sequence of BLCAP10 contains three LXXLL (where X represent any amino acid) motifs that are similar but not identical for both estrogen receptors. Therefore, the possibility of cross-reaction with the ERβ isoform should be taken into consideration. Computational analysis of the BLCAP10 mRNA sequence for the presence of potential transmembrane segments using “DAS” software revealed three transmembrane domains. The data of computational analysis confirmed the results of immunostaining experiments.

Primary analysis of BLCAP10 messenger RNA distribution pattern in human adult and foetal tissues was performed using RNA array technology. BLCAP10 messenger RNA expression levels were high in skeletal muscle, tongue, brain and cerebellum and lower levels were detectable in bladder and heart. It is still unknown whether BLCAP10 expression pattern is species dependent. The attempts to overexpress BLCAP10 in eukaryotic cells (HELA) failed eventually because of protein cytotoxicity. Moreover, even minor amounts of BLCAP10

produced in bacteria interfered with bacterial growth and/or inhibited protein synthesis. This observation was in the line with the data published by Zuo et al, who reported that HeLa cells expressing BLCAP10 showed reduced cell growth compared to the control cells. BLCAP10 expression in HeLa cells led also to growth arrest and significantly enhanced apoptosis in vitro and reduced tumor formation *in vivo* [176]. Further studies presented by the same research group reported that overexpression of BCAP10 resulted in growth inhibition of the human tongue cancer cell line Tca8113 in vitro, accompanied by S phase cell cycle arrest and apoptosis [177]. Despite its small size (10 kD), BLCAP10 protein possess three LXXLL domains that are protein-protein interaction interfaces characteristic for estrogen receptors, contains three transmembrane segments and, according to the results of immunostaining, could interact with estrogen receptor beta. Strong gene expression in non- dividing cells together with cytotoxic effects and/or protein expression inhibition suggested that BLCAP10 could play a pivotal role in cell cycle regulation processes in cardiac myocytes.

6. Outlook

In summary, both studies provide new insights into the role of estrogen receptors in the heart. Pharmacological approaches using estrogen receptor non- selective ligand 17β-estradiol and newly synthesised ER isoform selective ligands 16α-LE_2 (ERα agonist) and 8β- VE_2 (ERβ agonist) showed different function in the heart: ERα exerted its activity directly in the myocardium and upregulated αMHC expression whereas ERβ effectively lowered blood pressure and attenuated cardiac hypertrophy without influencing myosin expression. 8β-VE_2 could effectively regulate blood pressure without inducing uterine growth and those features make this substance interesting for biomedicine and pharmacological therapy. ERα selective ligand could be applied as an alternative to 17β- estradiol in hormone replacement therapy.

The second finding of this project was that 17β-estradiol as well as ER ligands were effective in young female rats but not in senescent rats. Moreover, the results showed much higher steroid hormone metabolism rates in senescent rats compared to young ones. Enzymes which metabolize estrogen are the members of hydroxysteroid dehydrogenase family. The following isozymes are involved in estrogen metabolism: 17β-HSD2, 17β-HSD4, 17β-HSD8 and 17β-HSD10. Aromatase, 17β-HSD1 and 17β-HSD7 are the major enzymes responsible for estrogen synthesis. Estrogen metabolism and synthesis are separated processes: enzymes participating in estrogen synthesis are localized in the female reproductive organs whereas the majority of enzymes that inactivate estrogen are localized in the liver. Therefore, further studies are required to determine estrogen metabolism rates in young and senescent rat liver samples, in particular 17β-HSD2 activity.

Another member of hydroxysteroid dehydrogenases, 17β-HSD10, was identified as one of the protein- protein interaction partners of estrogen receptor alpha in the heart cDNA library. Mitochondrial localization of both proteins and the functional relevance of 17β-HSD10 to estrogen metabolism make this interaction interesting. Of note, 17β-HSD10 and ERα interaction occurs only in the absence of 17β-estradiol or at very low levels of the hormone. Additional studies are required to determine critical estrogen concentrations that promote protein complex formation or its dissociation and functional consequences of this

interaction. Since 17β-HSD10 is localized only in mitochondria, one could expect that this enzyme is highly active in the organs containing substantial amounts of mitochondria, i.e. brain, liver and heart. According to the published data [178, 179], elevated expression of 17β- dehydrogenase 10 resulted in elevated estrogen metabolism and accelerated development of Alzheimer disease and stronger effects were observed in male individuals. Generation of 17β- dehydrogenase 10 knock-out mice would provide important information about the role of the enzyme under *in-vivo* conditions.

Generation of mouse knock-out model would also be interesting to elucidate the function of BLCAP10 *in vivo*. Current knowledge about tissue distribution and cellular localization of this protein provides first indications about expression pattern of this protein but little is known about its function and regulatory mechanisms.

This work showed that estrogen receptor action in heart is very complex which involves estrogen receptor dependent gene expression regulation, non- genomic action, estrogen metabolism and estrogen receptor interaction with other proteins. Therefore, further studies are required to understand estrogen receptor function in the heart and apply this knowledge for development of more powerful and effective treatment of cardiovascular disease in post- menopausal women.

7. Acknowledgements

I would like to express my sincere gratitude and appreciation to my advisor, Dr. med. Theo Pelzer for his support, patience and encouragement throughout my Ph.D. studies. He offered me so much advice, supervising me and always guiding me to the right direction. I really enjoyed interesting discussions and “brain storming” sessions throughout my PhD work. Special thanks are given to Prof. Dr. med. Ludwig Neyses who invited me, as a visiting scholar, to the laboratory of molecular cardiology at the University of Würzburg and later accepted me as a PhD student without any hesitation and gave me the unique opportunity to work in the research area of nuclear receptors. Many thanks to Prof. G. Ertl for the opportunity to work in the laboratory of molecular cardiology. I would like to thank Dr. Tertia de Jager who taught me most of the molecular biology methods used in our laboratory and who helped me to understand and interpret the results. I really admired sharp mind and experimental talent of Dr. Kai Schuh who will stay in my mind as an excellent scientist and a kind person. Paula- Anahi Arias- Loza, Natalie Burkard, Rongxue Wu, Matthias Hallhuber, Jenny Muck and Vijayakumar Govindaraj and Dr. Oliver Ritter for nice and fruitful collaboration, for tolerance and relaxed but creative atmosphere in the laboratory. I would also like to gratefully acknowledge Dr. Kai Hu and Charlotte Dienesch, Barbara Bayer and Helga Wagner for nice collaboration and for their commitment throughout the whole period of my work. I would like to express my deep sincere gratitude to Dr. Stepan Gambaryan for his help, support and long discussions. Many thanks to Dr. med. Michael Kreissl, Dr. Andreas Schirbel and Bernd Hallmann for an interesting collaboration in the field of positron emission tomography (PET).

I owe my thanks to my family. Without their encouragement and understanding it would have been impossible for me to finish this work.

8. References

1. Uemura K, Pisa Z. Trends in cardiovascular disease mortality in industrialized countries since 1950. *World Health Stat Q.* 1988;41(3-4):155-178.

2. Uemura K, Pisa Z. Recent trends in cardiovascular disease mortality in 27 industrialized countries. *World Health Stat Q.* 1985;38(2):142-162.

3. Kannel WB, Hjortland MC, McNamara PM, et al. Menopause and risk of cardiovascular disease: the Framingham study. *Ann Intern Med.* Oct 1976;85(4):447-452.

4. Howell SJ, Johnston SR, Howell A. The use of selective estrogen receptor modulators and selective estrogen receptor down-regulators in breast cancer. *Best Pract Res Clin Endocrinol Metab.* Mar 2004;18(1):47-66.

5. Hillard TC, Whitcroft S, Ellerington MC, et al. The long-term risks and benefits of hormone replacement therapy. *J Clin Pharm Ther.* Aug 1991;16(4):231-245.

6. Hulley S, Grady D, Bush T, et al. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *Jama.* Aug 19 1998;280(7):605-613.

7. Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *Jama.* Jul 17 2002;288(3):321-333.

8. Branca F. Dietary phyto-oestrogens and bone health. *Proc Nutr Soc.* Nov 2003;62(4):877-887.

9. Knight DC, Eden JA. Phytoestrogens--a short review. *Maturitas.* Nov 1995;22(3):167-175.

10. Ramsey LA, Ross BS, Fischer RG. Phytoestrogens and the management of menopause. *Adv Nurse Pract.* May 1999;7(5):26-30.

11. Garritano S, Pinto B, Giachi I, et al. Assessment of estrogenic activity of flavonoids from Mediterranean plants using an in vitro short-term test. *Phytomedicine.* Jan 2005;12(1-2):143-147.

12. Gerber M. Biofactors in the Mediterranean diet. *Clin Chem Lab Med.* Aug 2003;41(8):999-1004.

13. Pelzer T, Shamim A, Wolfges S, et al. Modulation of cardiac hypertrophy by estrogens. *Adv Exp Med Biol.* 1997;432:83-89.

14. Babiker FA, De Windt LJ, van Eickels M, et al. Estrogenic hormone action in the heart: regulatory network and function. *Cardiovasc Res.* Feb 15 2002;53(3):709-719.

15. Grohe C, Kahlert S, Lobbert K, et al. Cardiac myocytes and fibroblasts contain functional estrogen receptors. *FEBS Lett.* Oct 13 1997;416(1):107-112.

16. Faiman C, Winter JS, Reyes FI. Patterns of gonadotrophins and gonadal steroids throughout life. *Clin Obstet Gynaecol.* Dec 1976;3(3):467-483.

17. Aranda A, Pascual A. Nuclear hormone receptors and gene expression. *Physiol Rev.* Jul 2001;81(3):1269-1304.

18. Meier CA. Regulation of gene expression by nuclear hormone receptors. *J Recept Signal Transduct Res.* Jan-May 1997;17(1-3):319-335.

19. Couse JF, Korach KS. Reproductive phenotypes in the estrogen receptor-alpha knockout mouse. *Ann Endocrinol (Paris).* Jul 1999;60(2):143-148.

20. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev.* Jun 1999;20(3):358-417.

21. Emmen JM, Korach KS. Estrogen receptor knockout mice: phenotypes in the female reproductive tract. *Gynecol Endocrinol.* Apr 2003;17(2):169-176.

22. Jensen EV, DeSombre ER. Estrogen-receptor interaction. *Science.* Oct 12 1973;182(108):126-134.

23. Jensen EV, Desombre ER, Hurst DJ, et al. Estrogen-receptor interactions in target tissues. *Arch Anat Microsc Morphol Exp.* 1967;56(3):547-569.

24. Gosden JR, Middleton PG, Rout D. Localization of the human oestrogen receptor gene to chromosome 6q24----q27 by in situ hybridization. *Cytogenet Cell Genet.* 1986;43(3-4):218-220.

25. Nettles KW, Sun J, Radek JT, et al. Allosteric control of ligand selectivity between estrogen receptors alpha and beta: implications for other nuclear receptors. *Mol Cell.* Feb 13 2004;13(3):317-327.

26. Manas ES, Unwalla RJ, Xu ZB, et al. Structure-based design of estrogen receptor-beta selective ligands. *J Am Chem Soc.* Nov 24 2004;126(46):15106-15119.

27. Pratt WB. The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. *Annu Rev Pharmacol Toxicol.* 1997;37:297-326.

28. Pratt WB, Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev.* Jun 1997;18(3):306-360.

29. Glass CK. Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocr Rev.* Jun 1994;15(3):391-407.

30. Danielian PS, White R, Lees JA, et al. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *Embo J.* Mar 1992;11(3):1025-1033.

31. Parker MG. Structure and function of estrogen receptors. *Vitam Horm.* 1995;51:267-287.

32. Nichols M, Rientjes JM, Stewart AF. Different positioning of the ligand-binding domain helix 12 and the F domain of the estrogen receptor accounts for functional differences between agonists and antagonists. *Embo J.* Feb 2 1998;17(3):765-773.

33. Montano MM, Muller V, Trobaugh A, et al. The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Mol Endocrinol.* Jul 1995;9(7):814-825.

34. Kuiper GG, Enmark E, Pelto-Huikko M, et al. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A.* Jun 11 1996;93(12):5925-5930.

35. Tremblay GB, Tremblay A, Copeland NG, et al. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol Endocrinol.* Mar 1997;11(3):353-365.

36. Enmark E, Pelto-Huikko M, Grandien K, et al. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab.* Dec 1997;82(12):4258-4265.

37. Mosselman S, Polman J, Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.* Aug 19 1996;392(1):49-53.

38. Ruff M, Gangloff M, Wurtz JM, et al. Estrogen receptor transcription and transactivation: Structure-function relationship in DNA- and ligand-binding domains of estrogen receptors. *Breast Cancer Res.* 2000;2(5):353-359.

39. Lau YT. Receptor-dependent and genomic-independent actions of estrogen in vascular protection. *Chang Gung Med J.* Oct 2002;25(10):636-644.

40. Chambliss KL, Shaul PW. Estrogen modulation of endothelial nitric oxide synthase. *Endocr Rev.* Oct 2002;23(5):665-686.

41. Fernando RI, Wimalasena J. Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signaling through ERK and Akt. *Mol Biol Cell.* Jul 2004;15(7):3266-3284.

42. de Jager T, Pelzer T, Muller-Botz S, et al. Mechanisms of estrogen receptor action in the myocardium. Rapid gene activation via the ERK1/2 pathway and serum response elements. *J Biol Chem.* Jul 27 2001;276(30):27873-27880.

43. Coleman KM, Smith CL. Intracellular signaling pathways: nongenomic actions of estrogens and ligand-independent activation of estrogen receptors. *Front Biosci.* Oct 1 2001;6:D1379-1391.

44. Driggers PH, Segars JH. Estrogen action and cytoplasmic signaling pathways. Part II: the role of growth factors and phosphorylation in estrogen signaling. *Trends Endocrinol Metab.* Dec 2002;13(10):422-427.

45. Chen D, Pace PE, Coombes RC, et al. Phosphorylation of human estrogen receptor alpha by protein kinase A regulates dimerization. *Mol Cell Biol.* Feb 1999;19(2):1002-1015.

46. Picotto G, Massheimer V, Boland R. Acute stimulation of intestinal cell calcium influx induced by 17 beta-estradiol via the cAMP messenger system. *Mol Cell Endocrinol.* May 31 1996;119(2):129-134.

47. Sun M, Paciga JE, Feldman RI, et al. Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. *Cancer Res.* Aug 15 2001;61(16):5985-5991.

48. Simoncini T, Hafezi-Moghadam A, Brazil DP, et al. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature.* Sep 28 2000;407(6803):538-541.

49. Perks CM, Holly JM. Insulin-like growth factor binding proteins (IGFBPs) in breast cancer. *J Mammary Gland Biol Neoplasia.* Jan 2000;5(1):75-84.

50. Perks CM, Holly JM. IGFBPs and breast cancer. *Breast Dis.* 2003;17:91-104.

51. El-Tanani MK, Green CD. Interaction between estradiol and growth factors in the regulation of specific gene expression in MCF-7 human breast cancer cells. *J Steroid Biochem Mol Biol.* Mar 1997;60(5-6):269-276.

52. Marquez DC, Lee J, Lin T, et al. Epidermal growth factor receptor and tyrosine phosphorylation of estrogen receptor. *Endocrine.* Nov 2001;16(2):73-81.

53. Osborne CK, Schiff R, Fuqua SA, et al. Estrogen receptor: current understanding of its activation and modulation. *Clin Cancer Res.* Dec 2001;7(12 Suppl):4338s-4342s; discussion 4411s-4412s.

54. Ratajczak T. Protein coregulators that mediate estrogen receptor function. *Reprod Fertil Dev.* 2001;13(4):221-229.

55. Moggs JG, Orphanides G. Estrogen receptors: orchestrators of pleiotropic cellular responses. *EMBO Rep.* Sep 2001;2(9):775-781.

56. Margueron R, Duong V, Castet A, et al. Histone deacetylase inhibition and estrogen signalling in human breast cancer cells. *Biochem Pharmacol.* Sep 15 2004;68(6):1239-1246.

57. Davie JR, Moniwa M. Control of chromatin remodeling. *Crit Rev Eukaryot Gene Expr.* 2000;10(3-4):303-325.

58. Shang Y, Hu X, DiRenzo J, et al. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell.* Dec 8 2000;103(6):843-852.

59. Robyr D, Wolffe AP, Wahli W. Nuclear hormone receptor coregulators in action: diversity for shared tasks. *Mol Endocrinol.* Mar 2000;14(3):329-347.

60. Heery DM, Kalkhoven E, Hoare S, et al. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature.* Jun 12 1997;387(6634):733-736.

61. Beato M, Klug J. Steroid hormone receptors: an update. *Hum Reprod Update.* May-Jun 2000;6(3):225-236.

62. Razandi M, Oh P, Pedram A, et al. ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. *Mol Endocrinol.* Jan 2002;16(1):100-115.

63. Barletta F, Wong CW, McNally C, et al. Characterization of the interactions of estrogen receptor and MNAR in the activation of cSrc. *Mol Endocrinol.* May 2004;18(5):1096-1108.

64. Wong CW, McNally C, Nickbarg E, et al. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci U S A.* Nov 12 2002;99(23):14783-14788.

65. Song RX, Barnes CJ, Zhang Z, et al. The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. *Proc Natl Acad Sci U S A.* Feb 17 2004;101(7):2076-2081.

66. Sekeris CE. The mitochondrial genome: a possible primary site of action of steroid hormones. *In Vivo.* Sep-Oct 1990;4(5):317-320.

67. Monje P, Boland R. Subcellular distribution of native estrogen receptor alpha and beta isoforms in rabbit uterus and ovary. *J Cell Biochem.* 2001;82(3):467-479.

68. Chen JQ, Delannoy M, Cooke C, et al. Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells. *Am J Physiol Endocrinol Metab.* Jun 2004;286(6):E1011-1022.

69. Cammarata PR, Chu S, Moor A, et al. Subcellular distribution of native estrogen receptor alpha and beta subtypes in cultured human lens epithelial cells. *Exp Eye Res.* Apr 2004;78(4):861-871.

70. Yang SH, Liu R, Perez EJ, et al. Mitochondrial localization of estrogen receptor beta. *Proc Natl Acad Sci U S A.* Mar 23 2004;101(12):4130-4135.

71. Morkuniene R, Arandarcikaite O, Borutaite V. Estradiol prevents release of cytochrome c from mitochondria and inhibits ischemia-induced apoptosis in perfused heart. *Exp Gerontol.* Jul 2006;41(7):704-708.

72. Morkuniene R, Jekabsone A, Borutaite V. Estrogens prevent calcium-induced release of cytochrome c from heart mitochondria. *FEBS Lett.* Jun 19 2002;521(1-3):53-56.

73. Register TC, Adams MR. Coronary artery and cultured aortic smooth muscle cells express mRNA for both the classical estrogen receptor and the newly described estrogen receptor beta. *J Steroid Biochem Mol Biol.* Feb 1998;64(3-4):187-191.

74. Venkov CD, Rankin AB, Vaughan DE. Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function. *Circulation.* Aug 15 1996;94(4):727-733.

75. Grohe C, Kahlert S, Lobbert K, et al. Expression of oestrogen receptor alpha and beta in rat heart: role of local oestrogen synthesis. *J Endocrinol.* Feb 1998;156(2):R1-7.

76. Johnson BD, Zheng W, Korach KS, et al. Increased expression of the cardiac L-type calcium channel in estrogen receptor-deficient mice. *J Gen Physiol.* Aug 1997;110(2):135-140.

77. Yu W, Dahl G, Werner R. The connexin43 gene is responsive to oestrogen. *Proc Biol Sci.* Feb 22 1994;255(1343):125-132.

78. Weisz A, Bresciani F. Estrogen induces expression of c-fos and c-myc protooncogenes in rat uterus. *Mol Endocrinol.* Sep 1988;2(9):816-824.

79. Gupta MP, Gupta M, Zak R, et al. Egr-1, a serum-inducible zinc finger protein, regulates transcription of the rat cardiac alpha-myosin heavy chain gene. *J Biol Chem.* Jul 15 1991;266(20):12813-12816.

80. Nuedling S, Kahlert S, Loebbert K, et al. 17 Beta-estradiol stimulates expression of endothelial and inducible NO synthase in rat myocardium in-vitro and in-vivo. *Cardiovasc Res.* Aug 15 1999;43(3):666-674.

81. Mendelsohn ME. Protective effects of estrogen on the cardiovascular system. *Am J Cardiol.* Jun 20 2002;89(12A):12E-17E; discussion 17E-18E.

82. Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. *N Engl J Med.* Jun 10 1999;340(23):1801-1811.

83. Iafrati MD, Karas RH, Aronovitz M, et al. Estrogen inhibits the vascular injury response in estrogen receptor alpha-deficient mice. *Nat Med.* May 1997;3(5):545-548.

84. Karas RH, Hodgin JB, Kwoun M, et al. Estrogen inhibits the vascular injury response in estrogen receptor beta-deficient female mice. *Proc Natl Acad Sci U S A.* Dec 21 1999;96(26):15133-15136.

85. Taylor AH, Al-Azzawi F. Immunolocalisation of oestrogen receptor beta in human tissues. *J Mol Endocrinol.* Feb 2000;24(1):145-155.

86. Lou H, Martin MB, Stoica A, et al. Upregulation of estrogen receptor-alpha expression in rabbit cardiac allograft. *Circ Res.* Nov 2 1998;83(9):947-951.

87. Pelzer T, Jazbutyte V, Hu K, et al. The estrogen receptor-alpha agonist 16alpha-LE2 inhibits cardiac hypertrophy and improves hemodynamic function in estrogen-deficient spontaneously hypertensive rats. *Cardiovasc Res.* Sep 1 2005;67(4):604-612.

88. Couse JF, Lindzey J, Grandien K, et al. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Endocrinology.* Nov 1997;138(11):4613-4621.

89. Forster C, Kietz S, Hultenby K, et al. Characterization of the ERbeta-/-mouse heart. *Proc Natl Acad Sci U S A.* Sep 28 2004;101(39):14234-14239.

90. Zhu Y, Bian Z, Lu P, et al. Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. *Science.* Jan 18 2002;295(5554):505-508.

91. Miller WL. Molecular biology of steroid hormone synthesis. *Endocr Rev.* Aug 1988;9(3):295-318.

92. Kuiper GG, Lemmen JG, Carlsson B, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology.* Oct 1998;139(10):4252-4263.

93. Kurzer MS, Xu X. Dietary phytoestrogens. *Annu Rev Nutr.* 1997;17:353-381.

94. Harper MJ, Walpole AL. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. *J Reprod Fertil.* Feb 1967;13(1):101-119.

95. Clark JH, Peck EJ, Jr. Female sex steroids: receptors and function. *Monogr Endocrinol.* 1979;14:I-XII, 1-245.

96. Jordan VC. Third annual William L. McGuire Memorial Lecture. "Studies on the estrogen receptor in breast cancer"--20 years as a target for the treatment and prevention of cancer. *Breast Cancer Res Treat.* 1995;36(3):267-285.

97. Gutman M, Couillard S, Roy J, et al. Comparison of the effects of EM-652 (SCH57068), tamoxifen, toremifene, droloxifene, idoxifene, GW-5638 and raloxifene on the growth of human ZR-75-1 breast tumors in nude mice. *Int J Cancer.* May 10 2002;99(2):273-278.

98. Delmas PD, Bjarnason NH, Mitlak BH, et al. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. *N Engl J Med.* Dec 4 1997;337(23):1641-1647.

99. Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. *Jama.* Aug 18 1999;282(7):637-645.

100. Johnston CC, Jr., Bjarnason NH, Cohen FJ, et al. Long-term effects of raloxifene on bone mineral density, bone turnover, and serum lipid levels in early

postmenopausal women: three-year data from 2 double-blind, randomized, placebo-controlled trials. *Arch Intern Med.* Dec 11-25 2000;160(22):3444-3450.

101. Wakeling AE, Bowler J. ICI 182,780, a new antioestrogen with clinical potential. *J Steroid Biochem Mol Biol.* Sep 1992;43(1-3):173-177.

102. Dukes M, Miller D, Wakeling AE, et al. Antiuterotrophic effects of a pure antioestrogen, ICI 182,780: magnetic resonance imaging of the uterus in ovariectomized monkeys. *J Endocrinol.* Nov 1992;135(2):239-247.

103. Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, et al. Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. *J Natl Cancer Inst.* May 17 1995;87(10):746-750.

104. Meyer ME, Gronemeyer H, Turcotte B, et al. Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell.* May 5 1989;57(3):433-442.

105. Stauffer SR, Coletta CJ, Tedesco R, et al. Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *J Med Chem.* Dec 28 2000;43(26):4934-4947.

106. Katzenellenbogen BS, Choi I, Delage-Mourroux R, et al. Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. *J Steroid Biochem Mol Biol.* Nov 30 2000;74(5):279-285.

107. Sun J, Meyers MJ, Fink BE, et al. Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor-alpha or estrogen receptor-beta. *Endocrinology.* Feb 1999;140(2):800-804.

108. Hegele-Hartung C, Siebel P, Peters O, et al. Impact of isotype-selective estrogen receptor agonists on ovarian function. *Proc Natl Acad Sci U S A.* Apr 6 2004;101(14):5129-5134.

109. Meyers MJ, Sun J, Carlson KE, et al. Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J Med Chem.* Nov 22 2001;44(24):4230-4251.

110. Harris HA, Albert LM, Leathurby Y, et al. Evaluation of an estrogen receptor-beta agonist in animal models of human disease. *Endocrinology.* Oct 2003;144(10):4241-4249.

111. Hillisch A, Peters O, Kosemund D, et al. Dissecting physiological roles of estrogen receptor alpha and beta with potent selective ligands from structure-based design. *Mol Endocrinol.* Jul 2004;18(7):1599-1609.

112. August P, Oparil S. Hypertension in women. *J Clin Endocrinol Metab.* Jun 1999;84(6):1862-1866.

113. Burt VL, Cutler JA, Higgins M, et al. Trends in the prevalence, awareness, treatment, and control of hypertension in the adult US population. Data from the health examination surveys, 1960 to 1991. *Hypertension.* Jul 1995;26(1):60-69.

114. Burt VL, Whelton P, Roccella EJ, et al. Prevalence of hypertension in the US adult population. Results from the Third National Health and Nutrition Examination Survey, 1988-1991. *Hypertension.* Mar 1995;25(3):305-313.

115. Wallen WJ, Cserti C, Belanger MP, et al. Gender-differences in myocardial adaptation to afterload in normotensive and hypertensive rats. *Hypertension.* Nov 2000;36(5):774-779.

116. Dudley SD. Responsiveness to estradiol in central nervous system of aging female rats. *Neurosci Biobehav Rev.* Spring 1982;6(1):39-45.

117. Gaumet N, Braillon P, Seibel MJ, et al. Influence of aging on cortical and trabecular bone response to estradiol treatment in ovariectomized rats. *Gerontology.* 1998;44(3):132-139.

118. Williams JK, Suparto I. Hormone replacement therapy and cardiovascular disease: lessons from a monkey model of postmenopausal women. *Ilar J.* 2004;45(2):139-146.

119. Chapin RE, Stevens JT, Hughes CL, et al. Endocrine modulation of reproduction. *Fundam Appl Toxicol.* Jan 1996;29(1):1-17.

120. Fortepiani LA, Zhang H, Racusen L, et al. Characterization of an animal model of postmenopausal hypertension in spontaneously hypertensive rats. *Hypertension.* Mar 2003;41(3 Pt 2):640-645.

121. Xu Y, Arenas IA, Armstrong SJ, et al. Estrogen modulation of left ventricular remodeling in the aged heart. *Cardiovasc Res.* Feb 2003;57(2):388-394.

122. Schriefers H, Meyer SG, Bergheim E, et al. [Steroid hormone transformation in the gonads and liver of aged rats]. *Biol Chem Hoppe Seyler.* May 1988;369(5):371-380.

123. Carreno JE, Apablaza F, Ocaranza MP, et al. [Cardiac hypertrophy: molecular and cellular events]. *Rev Esp Cardiol.* May 2006;59(5):473-486.

124. Sadoshima J, Izumo S. The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu Rev Physiol.* 1997;59:551-571.

125. Okamoto K. [Experimental hypertension, especially on spontaneously hypertensive rats]. *Iryo.* Mar 1969;23(3):293-305.

126. Tanase H. Genetic control of blood pressure in spontaneously hypertensive rats (SHR). *Jikken Dobutsu.* Oct 1979;28(4):519-530.

127. Tanase H, Suzuki Y, Ooshima A, et al. Genetic analysis of blood pressure in spontaneously hypertensive rats. *Jpn Circ J.* Dec 1970;34(12):1197-1212.

128. Bing OH, Brooks WW, Conrad CH, et al. Intracellular calcium transients in myocardium from spontaneously hypertensive rats during the transition to heart failure. *Circ Res.* May 1991;68(5):1390-1400.

129. Krenz M, Sanbe A, Bouyer-Dalloz F, et al. Analysis of myosin heavy chain functionality in the heart. *J Biol Chem.* May 9 2003;278(19):17466-17474.

130. Lompre AM, Nadal-Ginard B, Mahdavi V. Expression of the cardiac ventricular alpha- and beta-myosin heavy chain genes is developmentally and hormonally regulated. *J Biol Chem.* May 25 1984;259(10):6437-6446.

131. Morkin E. Regulation of myosin heavy chain genes in the heart. *Circulation.* May 1993;87(5):1451-1460.

132. Pennock GD, Raya TE, Bahl JJ, et al. Combination treatment with captopril and the thyroid hormone analogue 3,5-diiodothyropropionic acid. A new approach to improving left ventricular performance in heart failure. *Circulation.* Sep 1993;88(3):1289-1298.

133. Weiss A, Leinwand LA. The mammalian myosin heavy chain gene family. *Annu Rev Cell Dev Biol.* 1996;12:417-439.

134. Alpert NR, Brosseau C, Federico A, et al. Molecular mechanics of mouse cardiac myosin isoforms. *Am J Physiol Heart Circ Physiol.* Oct 2002;283(4):H1446-1454.

135. He XY, Merz G, Yang YZ, et al. Function of human brain short chain L-3-hydroxyacyl coenzyme A dehydrogenase in androgen metabolism. *Biochim Biophys Acta.* Apr 12 2000;1484(2-3):267-277.

136. He XY, Schulz H, Yang SY. A human brain L-3-hydroxyacyl-coenzyme A dehydrogenase is identical to an amyloid beta-peptide-binding protein involved in Alzheimer's disease. *J Biol Chem.* Apr 24 1998;273(17):10741-10746.

137. Widder J, Pelzer T, von Poser-Klein C, et al. Improvement of endothelial dysfunction by selective estrogen receptor-alpha stimulation in ovariectomized SHR. *Hypertension.* Nov 2003;42(5):991-996.

138. Schultz Jel J, Witt SA, Glascock BJ, et al. TGF-beta1 mediates the hypertrophic cardiomyocyte growth induced by angiotensin II. *J Clin Invest.* Mar 2002;109(6):787-796.

139. He XY, Merz G, Mehta P, et al. Human brain short chain L-3-hydroxyacyl coenzyme A dehydrogenase is a single-domain multifunctional enzyme. Characterization of a novel 17beta-hydroxysteroid dehydrogenase. *J Biol Chem.* May 21 1999;274(21):15014-15019.

140. He XY, Yang SY, Schulz H. Cloning and expression of the fadH gene and characterization of the gene product 2,4-dienoyl coenzyme A reductase from Escherichia coli. *Eur J Biochem.* Sep 1 1997;248(2):516-520.

141. Gromova I, Gromov P, Celis JE. bc10: A novel human bladder cancer-associated protein with a conserved genomic structure downregulated in invasive cancer. *Int J Cancer.* Apr 1 2002;98(4):539-546.

142. Booth EA, Obeid NR, Lucchesi BR. Activation of estrogen receptor-alpha protects the in vivo rabbit heart from ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol.* Nov 2005;289(5):H2039-2047.

143. Kam KW, Qi JS, Chen M, et al. Estrogen reduces cardiac injury and expression of beta1-adrenoceptor upon ischemic insult in the rat heart. *J Pharmacol Exp Ther.* Apr 2004;309(1):8-15.

144. Kolodgie FD, Farb A, Litovsky SH, et al. Myocardial protection of contractile function after global ischemia by physiologic estrogen replacement in the ovariectomized rat. *J Mol Cell Cardiol.* Sep 1997;29(9):2403-2414.

145. Bush TL. Preserving cardiovascular benefits of hormone replacement therapy. *J Reprod Med.* Mar 2000;45(3 Suppl):259-273.

146. Bush TL. Evidence for primary and secondary prevention of coronary artery disease in women taking oestrogen replacement therapy. *Eur Heart J.* Aug 1996;17 Suppl D:9-14.

147. Anderson GL, Limacher M, Assaf AR, et al. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *Jama.* Apr 14 2004;291(14):1701-1712.

148. Oates MB, McGhan WF, Smith MD. Hormone replacement therapy: a review of the risk versus benefit--Part II. *Med Interface.* Jan 1997;10(1):108-114.

149. Kuttenn F, Gerson M. [Hormone replacement therapy of menopause, heart and blood vessels]. *Arch Mal Coeur Vaiss.* Jul 2001;94(7):685-689.

150. Heine PA, Taylor JA, Iwamoto GA, et al. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A.* Nov 7 2000;97(23):12729-12734.

151. Naaz A, Zakroczymski M, Heine P, et al. Effect of ovariectomy on adipose tissue of mice in the absence of estrogen receptor alpha (ERalpha): a potential role for estrogen receptor beta (ERbeta). *Horm Metab Res.* Nov-Dec 2002;34(11-12):758-763.

152. Mueller SO, Korach KS. Estrogen receptors and endocrine diseases: lessons from estrogen receptor knockout mice. *Curr Opin Pharmacol.* Dec 2001;1(6):613-619.

153. Nishino Y, Neumann F. Sialic acid content and organ weight of vagina and uterus as parameter for finding predominantly vaginotrophic or uterotrophic estrogens. *Acta Endocrinol Suppl (Copenh).* 1973;173:117.

154. Kerr MB, Marshall K, Senior J. A comparison of the uterotrophic response in cyclic and ovariectomized normotensive and spontaneously hypertensive rats. *J Endocrinol.* Nov 1992;135(2):263-269.

155. Couse JF, Korach KS. Contrasting phenotypes in reproductive tissues of female estrogen receptor null mice. *Ann N Y Acad Sci.* Dec 2001;948:1-8.

156. Levy D, Garrison RJ, Savage DD, et al. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med.* May 31 1990;322(22):1561-1566.

157. Babiker FA, De Windt LJ, van Eickels M, et al. 17beta-estradiol antagonizes cardiomyocyte hypertrophy by autocrine/paracrine stimulation of a guanylyl cyclase A receptor-cyclic guanosine monophosphate-dependent protein kinase pathway. *Circulation.* Jan 20 2004;109(2):269-276.

158. Sharkey LC, Holycross BJ, Park S, et al. Effect of ovariectomy and estrogen replacement on cardiovascular disease in heart failure-prone SHHF/Mcc- fa cp rats. *J Mol Cell Cardiol.* Aug 1999;31(8):1527-1537.

159. Miyata S, Minobe W, Bristow MR, et al. Myosin heavy chain isoform expression in the failing and nonfailing human heart. *Circ Res.* Mar 3 2000;86(4):386-390.

160. Chizzonite RA, Zak R. Regulation of myosin isoenzyme composition in fetal and neonatal rat ventricle by endogenous thyroid hormones. *J Biol Chem.* Oct 25 1984;259(20):12628-12632.

161. Chevalier B, Callens F, Charlemagne D, et al. Signal and adaptational changes in gene expression during cardiac overload. *J Mol Cell Cardiol.* Dec 1989;21 Suppl 5:71-77.

162. Simpson PC, Long CS, Waspe LE, et al. Transcription of early developmental isogenes in cardiac myocyte hypertrophy. *J Mol Cell Cardiol.* Dec 1989;21 Suppl 5:79-89.

163. Swynghedauw B. Developmental and functional adaptation of contractile proteins in cardiac and skeletal muscles. *Physiol Rev.* Jul 1986;66(3):710-771.

164. Schaub MC, Hefti MA, Harder BA, et al. Various hypertrophic stimuli induce distinct phenotypes in cardiomyocytes. *J Mol Med.* Nov-Dec 1997;75(11-12):901-920.

165. Carniel E, Taylor MR, Sinagra G, et al. Alpha-myosin heavy chain: a sarcomeric gene associated with dilated and hypertrophic phenotypes of cardiomyopathy. *Circulation.* Jul 5 2005;112(1):54-59.

166. Ihionkhan CE, Chambliss KL, Gibson LL, et al. Estrogen causes dynamic alterations in endothelial estrogen receptor expression. *Circ Res.* Nov 1 2002;91(9):814-820.

167. Robertson JA, Farnell Y, Lindahl LS, et al. Estradiol up-regulates estrogen receptor messenger ribonucleic acid in endometrial carcinoma (Ishikawa) cells by stabilizing the message. *J Mol Endocrinol.* Aug 2002;29(1):125-135.

168. Rune GM, Wehrenberg U, Prange-Kiel J, et al. Estrogen up-regulates estrogen receptor alpha and synaptophysin in slice cultures of rat hippocampus. *Neuroscience.* 2002;113(1):167-175.

169. Chu SH, Goldspink P, Kowalski J, et al. Effect of estrogen on calcium-handling proteins, beta-adrenergic receptors, and function in rat heart. *Life Sci.* Aug 22 2006;79(13):1257-1267.

170. Bupha-Intr T, Wattanapermpool J. Regulatory role of ovarian sex hormones in calcium uptake activity of cardiac sarcoplasmic reticulum. *Am J Physiol Heart Circ Physiol.* Sep 2006;291(3):H1101-1108.

171. Kravtsov GM, Kam KW, Liu J, et al. Altered Ca2+ handling by ryanodine receptor and Na+-Ca2+ exchange in the heart from ovariectomized rats - role of protein kinase A. *Am J Physiol Cell Physiol.* Dec 13 2006.

172. Cain BS, Meldrum DR, Joo KS, et al. Human SERCA2a levels correlate inversely with age in senescent human myocardium. *J Am Coll Cardiol.* Aug 1998;32(2):458-467.

173. Lakatta EG, Sollott SJ. Perspectives on mammalian cardiovascular aging: humans to molecules. *Comp Biochem Physiol A Mol Integr Physiol.* Aug 2002;132(4):699-721.

174. Solakidi S, Psarra AM, Sekeris CE. Differential subcellular distribution of estrogen receptor isoforms: localization of ERalpha in the nucleoli and ERbeta in

the mitochondria of human osteosarcoma SaOS-2 and hepatocarcinoma HepG2 cell lines. *Biochim Biophys Acta.* Sep 30 2005;1745(3):382-392.

175. Peltoketo H, Luu-The V, Simard J, et al. 17beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. *J Mol Endocrinol.* Aug 1999;23(1):1-11.

176. Zuo Z, Zhao M, Liu J, et al. Functional analysis of bladder cancer-related protein gene: a putative cervical cancer tumor suppressor gene in cervical carcinoma. *Tumour Biol.* 2006;27(4):221-226.

177. Yao J, Duan L, Fan M, et al. Overexpression of BLCAP induces S phase arrest and apoptosis independent of p53 and NF-kappaB in human tongue carcinoma : BLCAP overexpression induces S phase arrest and apoptosis. *Mol Cell Biochem.* Oct 10 2006.

178. He XY, Wen GY, Merz G, et al. Abundant type 10 17 beta-hydroxysteroid dehydrogenase in the hippocampus of mouse Alzheimer's disease model. *Brain Res Mol Brain Res.* Feb 28 2002;99(1):46-53.

179. Wen GY, Yang SY, Kaczmarski W, et al. Presence of hydroxysteroid dehydrogenase type 10 in amyloid plaques (APs) of Hsiao's APP-Sw transgenic mouse brains, but absence in APs of Alzheimer's disease brains. *Brain Res.* Nov 1 2002;954(1):115-122.

9. Appendix

9.1. Abbreviations

16α-LE_2	3.17-dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-21,16α-lactone
17β-HSD10	17β-hydroxysteroid dehydrogenase type 10
8β-VE_2	8-vinylestra-1,3,5(10)-triene-3,17β-diol
A	absorbance
aa	Amino acids
absBW	Absolute body weight
absHW	Absolute heart weight
adiol	3α-androstanediol
Amp^r	Ampicillin resistance
AP-1	Transcription factor AP-1
APS	Ammonium persuphate
ASD	Androstenedione
ATP	Adenosine triphosphate
b	Path length
BamHI	restriction enzyme
BERKO	Estrogen receptor beta knockout mouse
BL21	E.coli strain, also called DE3
BLCAP10	Bladder cancer associated protein 10
bp	Base pairs
BSA	Bovine serum albumin
c	Analyte concentration
cDNA	Complementary DNA
CMV	cytomegalovirus
CO	Cardiac output
COX I	Cytochrome oxidase I
DAP	Diastolic aortic pressure
DEPC	Diethyl pyrocarbonate
DH5α	E.coli strain
DHEA	Dihydroepiandrosterone
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Desoxyribonuleic acid
dNTP	Desoxyribonucleotide triphosphates
dp/dt	Pressure change during the time
E. coli	Escherichia coli
E1	Estrone
E2	17β-estradiol
E3	Estriol
ECL	Enhance chemiluminescence reagent
EcoRI	Restriction endonuclease form E. coli
EGTA	Ethylene glycol- bis (β- aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
ERK 1/2	Extracellular signal- regulated kinase
ERKO	Estrogen receptor alpha knockout mouse
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta

FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione-S-transferase
HELA	Human cervix carcinoma cells
HEPES	N-2-hydroxyethylpiperazin-N'-2'-ethansulfonacid
His	histidine
HR	Heart rate
HRP	Horse reddish peroxidase
IGEPAL CA-630	Octylphenyl-polyethylene glycol
IPTG	Isopropyl β-D-thiogalactopyranoside
K_m	Michaelis constant
ko	Knockout
lacZ	Beta galactosidase reporter gene
LB medium	Luria- Bertani medium
LBD	Lingand binding domain
LV	Left ventricle
LVEDP	Left ventricle end-diastolic pressure
LVSP	Left ventricle systolic pressure
MAP	Mean aortic pressure
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	Messenger RNA
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	Sodium hydroxide
NCBI	National Center of Biotechnology Information
OD	Optical density
ovx	Ovariectomy
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pBT	Bait vector
PCR	Polymerase chain reaction
pDONR201	Donor vector
PLB	phospholamban
PMSF	Phenylmethylsulfonyl fluoride
pPLB	Phosphorylated phospholamban
pTRG	Target vector
relHW	Relative heart weight
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation buffer
RNA	Ribonucleic acid
RNAP alpha	RNA polymerase α subunit
rRNA	Ribosomal RNA
RS	Reporter strain competent cells
RT-PCR	Reverse transcription- polymerase chain reaction
[S]	Substrate concentration
S.O.C. medium	Super optimal broth for catabolite repression
sc	Subcutaneous injection

SDS	Sodium dodecylsulphate
SEM	Standard error of the mean
SERCA2	Sarcoplasmic reticulum calcium ATPase 2
SHR	Spontaneously hypertensive rats
SRE	Serum response element
SSC	Sodium chloride/ sodium citrate buffer
T free	Free testosterone
T total	Total testosterone
T4 ligase	Ligation enzyme from bacteriophage T4
T7	Bacteriophage T7 promoter
TAE	Tris Acetate-EDTA buffer
Tris	Trishydroxymethylaminomethan
UV	ultraviolet
V	Reaction velosity
v_{max}	Maximal reaction velocity
v_o	Initial velocity phase
vol	Volume
wt	Wild type
α/β MHC	alpha/ beta myosin heavy chain
βME	β- mercaptoethanol
ε	Extinction coefficient
λCI	Bacteriophage λ repressor protein
BrdU	Bromdesoxyuridine

9.2. Suppliers of materials

16α-LE_2	Schering AG
17β- estradiol	Sigma
8β-VE_2	Schering AG
Agarose	Sigma
Agarose, low melting temperature	Invitrogen
Ampicillin	Sigma
APS	Sigma
BamHI	Fermentas
BL21	Invitrogen
Bradford protein assay	BioRad
BSA	Sigma
Chracoal stripped FCS	cc Pro
Complete EDTA-free protease inhibitor cocktail tablets	Roche
DEPC	Sigma
DH5α cells	
DMEM	Sigma
ECL western blotting detection reagents	Amersham- GE Helthcare
EcoRI	Fermentas
EDTA	Sigma
EGTA	Sigma
FCS	PAA
Formaldehyde	Merck
Formamide	Merck
Glutathione sepharose ™4B	Amersham Pharmacia
Glycerol	Merck
Glycine	Merck/Serva
HELA cells	DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen
HEPES	Sigma
ICI 182780	Tocris
IGEPAL CA-630	Sigma
IPTG	Sigma
L-(+)-Arabinose	Sigma
LB-agar	Sigma
Minimal essential medium (MEM)	Sigma, Biozol
MOPS	Sigma
NAD^+	Sigma
NADH	Sigma
Ni-NTA agarose	Qiagen
Peanut oil	Sigma
Peptone	Sigma
Plasmid DNA Maxi, Midi and Mini Prep kit	Macherey and Nagel
PMSF	Sigma
Protran nitrocellulose transfer membrane	Whatmann Schleicher & Schuell
Quick gel extraction kit	Qiagen

Radioimmunoassay kits	Diagnostic system laboratories (DSL)
RS	Invitrogen
S.O.C. medium	Invitrogen
SDS	Sigma
SDS	Sigma
T4 ligase	Fermentas
Tris	Merck
Tris base	Merck
Trizol reagent	Invitrogen
Trypsin	Sigma
Vimentin (Heparin)	Roche
Yeast extract	Sigma
βME	Merck

9.3. Buffers and solutions

9.3.1. Neonatal rat cardiomyocyte preparation:

CBFHH (Calcium and bicarbonate- free Hanks with Hepes)

NaCl	8 g
KCl	0.4 g
$MgSO_4 \cdot 7H_20$	0.2 g
Dextrose	1 g
KH_2PO_4	0.06 g
Na_2HPO4 anhydrogenous	0.048 g
HEPES	4.77 g
H_2O	to 1 L
pH	7.4

T&D (Trypsin and DNase)

Add 1.5 mg/ml Trypsin and 1 ml of 2mg/ml DNase dissolved in 0.15M NaCl per 100 ml of CBFHH.

MEM/5, 1 L

BrdU (20 stock solution)	5 ml
Vitamin B12 (2 mg/ml stock solution)	1 ml
Penicillin (782 mg dissolved in 25 ml)	1 ml
Streptomycin (50 mg/ml stock solution)	1 ml
charcoal stripped FCS	50 ml

9.3.2. RNA

10x MOPS (RNA running buffer)

MOPS	41.86 g
$NaOAc \cdot 3H_2O$	6.8 g
Na_4EDTA (Titriplex)	3.8 g
H_20	1 L

RNA agarose gel (containing formaldehyde)

Low- melting temperature agarose	1 g
DEPC- treated water	88 ml
10x MOPS buffer	10 ml
Ethidium bromide (EtBr)	1 µl
Formaldehyde	1.87 ml

RNA loading dye

Formamide	720 µl
10x MOPS	160 µl
Formaldehyde	260 µl

H_2O	193 µl
6x Bromphenol blue DNA loading dye	267 µl

9.3.3. Western blotting

RIPA buffer

1x PBS	97.5 ml
1% Igepal CA-630	1 ml
0.5% Sodium dexoxycholic acid	500 mg
0.1% SDS	1 ml of 10% stock solution

5x Electrophoresis buffer

Tris- base	15 g
Glycin	72 g
SDS	5 g
H_2O	To 1 L
pH	8.3

1x Transfer buffer

5x Electrophoresis buffer	200 ml
Methanol	200 ml
H_2O	600 ml

5x PBS

Na_2HPO_4	41.17 g
NaH_2PO_4	11.73 g
NaCl	20 g
H_2O	1 L
pH	7.4

PBS/Tween 20

1x PBS	To 1 L
0.05% Tween 20	500 ml

Separating polyacrylamide gels

	5%	***7.5%***	***10%***	***12%***	***15%***
H_2O	5.86 ml	4.85 ml	4 ml	3.35 ml	2.35 ml
1.5 M Tris pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% SDS	100 µl	100 µl	100 µl	100 µl	100 µl
Acrylamide/ Bis (30: 2)	1.66 ml	2.5 ml	3.33 ml	4 ml	5 ml
10% APS	50 µl				
TEMED	5 µl				

Stacking gel (5%)

	For 1 gel	***For 2 gels***
H_2O	2.85 ml	5.7 ml
0.5 M Tris pH 6.8	1.25 ml	2.5 ml
10% SDS	50 µl	100 µl
Acrylamide/ Bis (30: 2)	850 µl	1.7 ml
10% APS	25 µl	50 µl
TEMED	5 µl	10 µl

9.3.4. α/β myosin heavy chain (α/β MHC) electrophoresis

MHC sample buffer

0.3 M Na Cl	1.75 g
0.1 M NaH_2PO_4· H20	1.38 g
50 mM Na_2HPO_4	0.71 g
10 mM Na pyrophosphate	0.446 g
1 mM $MgCl \cdot 6H_2O$	0.02 g
10 mM EDTA	0.37 g
H_2O	to 100 ml
pH	7.4
25x Protease Inhibitor coctail	

8x Electrophoresis buffer

0.2 M Tris pH 8.3	24.22 g
1.54 M Glycin	115.8 g
0.8% SDS	80 ml
H_2O	to 1 L
pH	8.3

Upper phase buffer

1x Electrophoresis buffer	125 ml of 8x Electrophoresis buffer
β- mercaptoethanol	1.25 ml
H_2O	to 1 L

6% SDS- Glycine gel containing 5% Glycerol

	6% separating gel	4% stacking gel
100% glycerol	1 ml	0.5 ml
23% Acrylamide/Bis (50:1)	5.3 ml	1.7 ml
1.5 ml Tris (pH 8.8)	2.7 ml	----------
0.5 ml Tris (pH 6.8)	----------	1.4 ml
1 M Glycine	2 ml	----------
0.5 M EDTA (pH 8.0)	----------	80 µM
10% SDS	800 µl	400 µl
H_20	8 ml	5.8 ml
10 % APS	200 µl	100 µl
TEMED	10 µl	5 µl

Bacterial growth medium LB

NaCl	10 g
Yeast extract	5 g
Peptone	10 g
H_2O	to 1 L

Electrophoresis buffers:

10xTBE:

Tris base	108 g
Boric acid	55 g
0.5 M EDTA (pH 8.0)	40 ml
H_20	To 1 L

50x TAE:

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
H_20	To 1 L

10. Curriculum Vitae

Name	**Virginija Jazbutyte**
Address for correspondence	Wagnerstr. 3a, 97080 Würzburg, Germany Tel. 09312-2994337
Date and place of birth	10. 03.1972 Skuodas, Lithuania
Nationality/ citizenship	Lithuanian
Gender	Female
Primary and secondary education	09.1979- 06.1990, Skuodas Secondary school Nr.1, Skuodas, Lithuania
Higher education	09.1991- 06.1995, Bachelor qualification degree in biology, Vytautas Magnus university, Kaunas, Lithuania. Subject of diploma thesis/dissertation: The state of intracardiac nervous system of Daubentons's bat (Myotis daubentoni) during hibernation". Name of adviser: Prof. Dr. Dainius H. Pauza. Final grade: 9, Very good 09.1995- 06.1997, Master qualification degree in molecular biology and biotechnology, Vytautas Magnus university, Kaunas, Lithuania. Subject of diploma thesis/dissertation: Morphological analysis of hibernant heart. Comparison of natural and pathological hibernation. Name of adviser: Dr. Neringa Pauziene. Final grade: 9, Very good 09.1998- 05.1999, PhD studies in Biochemistry, Vytautas Magnus university,

	Kaunas, Lithuania
	10.1999- 04.2006, PhD studies (Promotion), Julius- Maximilians university, Würzburg, Germany
Scholarships	PhD scholarship (Promotionsstipendium), German Academical Exchange Service (DAAD) Research scholarship from University Clinics in Wuerzburg
Professional experience	09.1997- 06.1998, Engineer, Vytautas Magnus university, Kaunas, Lithuania.
Current occupation	01.05.2006- until now, biologist in the Clinics and Policlinics of Anesthesiology, University clinics of Würzburg, Germany.

11. List of publications:

Arias-Loza PA, **Hu K**, **Schafer A**, **Bauersachs J**, **Quaschning T**, **Galle J**, **Jazbutyte V**, **Neyses L**, **Ertl G**, **Fritzemeier KH**, **Hegele-Hartung C**, **Pelzer T**.
Medroxyprogesterone acetate but not drospirenone ablates the protective function of 17 beta-estradiol in aldosterone salt-treated rats.
Hypertension. 2006 Nov;48(5):994-1001.

Jazbutyte V, **Hu K**, **Kruchten P**, **Bey E**, **Maier SK**, **Fritzemeier KH**, **Prelle K**, **Hegele-Hartung C**, **Hartmann RW**, **Neyses L**, **Ertl G**, **Pelzer T**.
Aging reduces the efficacy of estrogen substitution to attenuate cardiac hypertrophy in female spontaneously hypertensive rats.
Hypertension. 2006 Oct;48(4):579-86.

Pelzer T, Jazbutyte V, Hu K, Segerer S, Nahrendorf M, Nordbeck P, Bonz AW, Muck J, Fritzemeier KH, Hegele-Hartung C, Ertl G, Neyses L.
The estrogen receptor-alpha agonist 16alpha-LE2 inhibits cardiac hypertrophy and improves hemodynamic function in estrogen-deficient spontaneously hypertensive

rats.
Cardiovasc Res. 2005 Sep 1;67(4):604-12.

Pelzer T, Jazbutyte V, Arias-Loza PA, Segerer S, Lichtenwald M, Law MP, Schafers M, Ertl G, Neyses L.
Pioglitazone reverses down-regulation of cardiac PPARgamma expression in Zucker diabetic fatty rats.
Biochem Biophys Res Commun. 2005 Apr 8;329(2):726-32.

Widder J, Pelzer T, von Poser-Klein C, Hu K, Jazbutyte V, Fritzemeier KH, Hegele-Hartung C, Neyses L, Bauersachs J.
Improvement of endothelial dysfunction by selective estrogen receptor-alpha stimulation in ovariectomized SHR.
Hypertension. 2003 Nov;42(5):991-6. Epub 2003 Oct 27.

Nahrendorf M, Frantz S, Hu K, von zur Muhlen C, Tomaszewski M, Scheuermann H, Kaiser R, Jazbutyte V, Beer S, Bauer W, Neubauer S, Ertl G, Allolio B, Callies F.
Effect of testosterone on post-myocardial infarction remodeling and function.
Cardiovasc Res. 2003 Feb;57(2):370-8.

Pelzer T, Neumann M, de Jager T, Jazbutyte V, Neyses L.
Estrogen effects in the myocardium: inhibition of NF-kappaB DNA binding by estrogen receptor-alpha and -beta.
Biochem Biophys Res Commun. 2001 Sep 7;286(5):1153-7.