Estrogen action in the myocardium: modulation of myocardial gene expression and the influence on cardiac hypertrophy

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

vorgelegt von

Tertia de Jager

Somerset West, Südafrika

Würzburg, 2002

Eingereicht am: 26. November 2001

Mitglieder der Promotionskommission:

Vorsitzender:

Gutachter: Prof. Dr. B. Allolio

Gutachter: Prof. Dr. K. Scheller

Tag des Promotionskolloquiums: 10. April 2002

Doktorurkunde ausgehändigt am:

Table of Contents:

Erklärung

Erklärung gemäß § 4 Abs. 3 der Promotionsordnung

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutz habe.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe bisher außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Graden erworben oder zuerwerben versucht.

Würzburg, 23. November 2001

Summary

Cardiovascular disease is the leading cause of mortality in both men and women in the Western world. Earlier observations have pointed out that pre-menopausal women have a lower risk of developing cardiovascular disease than age-matched men, with an increase in risk after the onset of menopause. This observation has directed the attention to estrogen as a potential protective factor in the heart. So far the focus of research and clinical studies has been the vascular system, leaving the current knowledge on the role of estrogen in the myocardium itself rather scarce. Functional estrogen receptor-α as well as -β have recently been identified in the myocardium, making the myocardium an estrogen target organ.

The focus of this thesis was 1) to investigate the role of estrogen and estrogen receptors in modulating myocardial gene expression both *in vivo* in an animal model for cardiac hypertrophy (spontaneously hypertensive rats; SHR), as well as *in vitro* in isolated neonatal cardiomyocytes, 2) to investigate the mechanisms of the rapid induction of an estrogen target gene, the early growth response gene-1 (Egr-1) and 3) to initiate the search for novel estrogen target genes in the myocardium.

1) The effects of estrogen on the expression of one of the major myocardial specific contractile proteins, the α-myosin heavy chain (α-MHC) have been investigated. In ovarectomised animals treated either with 17β-estradiol alone or in combination with a specific estrogen receptor antagonist, ICI 182780, it was shown that both αMHC mRNA and protein were upregulated by estrogen in an estrogen receptor specific manner. The *in vivo* results were confirmed *in vitro* in isolated neonatal cardiomyocytes which showed that estrogen has a direct action on the myocardium potent enough to upregulate the expression of α-MHC. Furthermore it was shown that the α-MHC promoter is induced by estrogen in an estrogen receptor-dependent manner and first investigations into the mechanisms involved in this upregulation identified Egr-1 as a potential transcription factor which, upon induction by estrogen, drives the expression of the α-MHC promoter.

2) Previously it was shown that Egr-1 is rapidly induced by estrogen in an estrogen receptor-dependent manner which was mediated via 5 serum response elements (SREs) in

the promoter region and surprisingly not via the estrogen response elements (EREs). In this study it was shown that estrogen-treatment of cardiomyocytes resulted in the recruitment of serum response factor (SRF), or an antigenically related protein, to the SREs in the Egr-1 promoter, which was specifically inhibited by the estrogen receptor antagonist ICI 182780. Transfection experiments showed that estrogen induced a heterologous promoter consisting only of 5 tandem repeats of the c-fos SRE in an ER-dependent manner, which identified SREs as promoter elements able to confer an estrogen response to target genes.

3) Potentially new target genes regulated by estrogen *in vivo* were analysed using hearts of ovarectomised animals as well as ovarectomised animals treated with estrogen. Analyses of cDNA microarray filters containing 1250 known genes identified 24 genes that were modified by estrogen *in vivo*. Among these genes, some might have potentially important functions in the heart and further analyses of these genes will create a more global picture of the role and function of estrogen in the myocardium.

Taken together, the results showed that estrogen does have a direct action on the myocardium both by regulating the expression of myocardial specific genes *in vivo*, as well as exerting rapid non-nuclear effects in cardiac myocytes. It was shown that SREs in the promoter region of genes can confer an estrogen response to genes identifying SREs as important elements in regulation of genes by estrogen. Furthermore, 24 potentially new estrogen targets were identified in the myocardium, contributing to the general understanding of estrogen action in the myocardium.

Zusammenfassung

Kardiovaskuläre Erkrankungen gehören zu den häufigsten Todesursachen in westlichen Ländern. Vor den Wechseljahren besteht für Frauen ein geringeres Risiko, Herzerkrankungen zu entwickeln als für gleichaltrige Männer. Nach dem Eintritt der Menopause sind diese geschlechtsspezifischen Unterschiede aufgehoben, da sich das Risiko bei Frauen erhöht. Dies deutet darauf hin, dass Östrogene kardioprotektiv wirken. Bislang konzentrierten sich sowohl Forschung als auch klinische Studien hauptsächlich auf das vaskuläre System, so dass wenig über die Rolle von Östrogenen im Myokard bekannt ist. Zwar wurde gezeigt, dass funktionelle Östrogenrezeptoren (α- und β-Form) in Herzmuskelzellen exprimiert werden, ihre genaue Funktion im Myokard ist jedoch noch ungeklärt.

Dies lieferte den Ansatzpunkt für die vorliegende Arbeit, die sich mit der Rolle von Östrogenen im Myokard beschäftigte. Dazu wurde 1) der Einfluss der Östrogene auf die Genexpression im Myokard sowohl *in vivo* mit Hilfe eines Tiermodells für Herzhypertrophie, der spontan hypertensiven Ratte (SHR), als auch *in vitro* an isolierten neonatalen Kardiomyozyten untersucht. 2) Weiterhin wurde der Mechanismus der schnellen Geninduktion durch Östrogene mit Hilfe eines bereits identifizierten östrogenresponsiven Gens, dem „Early growth response gene-1“ (Egr-1), analysiert. 3) Gleichzeitig wurden erste Versuche zur Identifizierung bislang unbekannter Östrogenzielgene im Myokard eingeleitet.

1) Die Beeinflussung der myokardiale Genexpression wurde an Hand eines myokardial spezifischen kontraktilen Proteins, der α-Myosinschwerkette (α-MHC), untersucht. Östrogensubstitution in ovarektomierten Ratten induzierte α-MHC sowohl auf mRNA- als auch auf Protein-Ebene in einer spezifisch durch Östrogenrezeptoren vermittelten Weise. In isolierten Kardiomyozyten konnten diese *in vivo* Ergebnisse bestätigt werden, indem gezeigt wurde, dass Östrogene direkt auf die Herzmuskelzellen wirken und die α-MHC-Expression induzieren. Weiterhin wurde die Östrogenrezeptor-abhängige Induktion des α-MHC-Promotors durch Östrogene nachgewiesen. Untersuchungen zu dem Mechanismus dieser

Induktion identifizierten Egr-1 als potentiellen Transkriptionsfaktor, der durch Östrogene induziert wird und so die α-MHC-Induktion vermittelt.

2) Es wurde bereits in einer früheren Arbeit nachgewiesen, dass Egr-1 durch Östrogene in Kardiomyozyten induziert wird. Diese schnelle Induktion wird durch serumresponsive Elemente (SREs), nicht aber durch die östrogenresponsiven Elemente (EREs) im Egr-1 Promotor vermittelt. Zur Untersuchung des Mechanismus dieser schnellen Induktion konnte in der vorliegenden Arbeit gezeigt werden, dass Östrogenbehandlung die Bindung von SRF (serum response factor) an die SREs im Egr-1 Promoter auslöst. Ebenso wurde ein künstlicher Promotor aus 5 SREs des c-fos Promotors durch Östrogene induziert. Diese Induktion fand in einer spezifisch Östrogenrezeptor-vermittelten Weise statt. Damit konnten SREs neben EREs und AP-1 als Promoterelemente identifiziert werden, die eine wichtige Rolle bei der östrogenabhängigen Induktion von Zielgenen spielen.

3) Ein Ansatz zur Identifizierung neuer potentieller Östrogenzielgene im Myokard wurde etabliert. Mit Hilfe der Microarray-Technik wurde die Genexpression in Herzen von ovarektomierten, östrogenbehandelten Tieren mit unbehandelten Tieren verglichen. Durch die Verwendung von cDNA Mikroarray-Membranen mit 1250 cDNAs bekannter Rattengene wurden 24 Gene identifiziert, deren Expression möglicherweise durch Östrogene im Herzen beeinflusst werden könnte.

Im Rahmen dieser Arbeit ist es somit gelungen zu zeigen, dass Östrogene eine direkte Wirkung auf das Myokard haben, indem sie die Expression des wichtigen kontraktilen Proteins α-MHC beeinflussen. Daneben vermitteln Östrogene auch schnelle nicht-genomische Geninduktionen via SREs. Zusätzlich wurden 24 potentiell neue Östrogenzielgene im Myokard identifiziert, was zu dem allgemeinen Verständnis der Östrogenwirkung im Myokard beiträgt.

Abbreviations

ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BNP	brain natriuretic peptide
BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine
cDNA	complementary DNA
CPSR1	controlled process serum replacement-1
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E_2	17β-estradiol
ECL	enhanced chemiluminescence
EGTA	ethylene glycol bis (β-aminoethyl ether) N, N, N', N' – tetraacetic acid
EDTA	ethylene disodium N, N, N', N' – tetraacetic acid
Egr-1	Early growth response gene-1
EMSA	electrophoretic mobility shift assay
eNOS	endothelial nitric oxide synthase
ER	Estrogen receptor
ERE	Estrogen response element
ERK1/2	extracellular signal-regulated kinase 1/2
FCS	Fetal calf serum
HEPES	N- (2-hydroxyethyk) piperazine – N' – (2-ethanesulfonic acid)
HRT	Hormone replacement therapy

ICI	ICI 182780, estrogen receptor antagonist
MAPK	mitogen activated protein kinase
MEM	minimal essential medium
MHC	Myosin heavy chain
MOPS	3- (N-morpholino) propanesulfonic acid
mRNA	messenger RNA
Ovx	Ovarectomy
PMA	phorbol-myristate-acetate
RIA	Radio immuo assay
RNA	ribonucleic acid
RPA	RNase protection assay
SDS	sodium dodecyl sulfate
SERMs	selective estrogen receptor modulators
SHR	spontaneously hypertensive rat
SRE	Serum response element
SRF	Serum response factor
T3	3,3',-triiodo-$_L$-thionine, thyroid hormone
TCF	ternary complex factor
tRNA	transfer RNA

1 Introduction

1.1 Gender specific differences in the development of heart disease

Cardiovascular disease remains the leading cause of mortality in the western world. Many studies have previously focussed on men, because of the high mortality due to cardiac disease in that gender. Recently it has been recognised that cardiovascular disease also affects women to a great extent and is the most frequent cause of female death in industrial countries. However, significant gender differences exist in both the development of cardiac disease as well as cardiac function and physiology (Hayward et al., 2000). Early observations from the Framingham study have directed attention to the fact that pre-menopausal women have a better prognosis after onset of congestive heart failure than age-matched men, but that there was no significant difference in the prognosis after congestive heart failure in older post-menopausal women and age-matched men (Ho et al., 1993). This observation lead believe that estrogen plays a protective role in the female heart and has become the basis for several estrogen therapy attempts in the treatment of cardiovascular disease. The mechanisms of this cardioprotective effect are not completely understood and have become the topic of controversial discussions as well as extensive research during the last few years.

1.2 The role of estrogen in the development and prevention of heart disease in women

Studies investigating the role of estrogen in the prevention of heart disease have so far mainly focussed on the role of hormone replacement therapy (HRT) on the vasculature (reviewed in Mendelsohn and Karas, 1999). It has been observed that the incidence of coronary artery disease is low in premenopausal women, rises in postmenopausal women, but is reduced to premenopausal levels when postmenopausal women receive estrogen therapy (Barrett-Connor and Bush, 1991; Grady et al., 1992; Stampfer et al., 1991). These observations used to be attributed to an estrogen-dependent lowering of serum lipid levels, however the protective effects of estrogen on serum lipid concentrations could not account

for the magnitude of the observed clinical benefits of estrogen (Bush et al., 1987; Mendelsohn and Karas, 1994). A direct estrogen action on the blood vessels has thus been suggested to play a substantial role in the cardioprotective effects of estrogen (Farhat et al., 1996; Mendelsohn and Karas, 1994). It has for example been observed that estrogen-treatment resulted in vasodilatation and inhibited the development of atherosclerotic lesions (Gilligan et al., 1994; Guetta et al., 1997; Lieberman et al., 1994; Reis et al., 1994; Sudhir et al., 1997). Vasodilatation occurs within 5 to 20 minutes of estrogen-treatment and is not dependent on changes in gene expression. These rapid estrogen actions have been referred to as “non-genomic” estrogen actions. The estrogen-induced prevention of atherosclerotic plaque formation occurs over a period of hours or days after estrogen-treatment and are dependent on changes in vascular gene expression and are therefore referred to as “genomic” estrogen actions. Investigations into the mechanisms of estrogen actions, especially in understanding the mechanisms of rapid non-genomic as well the slower genomic actions have been a topic of intensive research during the last few years. The role of estrogen receptors in these mechanisms of action has also been extensively investigated and is briefly reviewed in the next section.

1.3 Mechanisms of estrogen receptor action

Estrogens are female sex steroid hormones mainly produced in the ovaries, testis and adipose tissue. They diffuse in and out of cells where they are retained by specific estrogen receptors. Binding of estrogens to their receptors results in a conformational change in the receptor and thus activation of the ER. Cloning of the estrogen receptor gene in 1986 has shed light on the structure and molecular mechanism of action of estrogens and estrogen receptors (Green et al., 1986). In 1997, a second estrogen receptor has been identified and cloned and was termed estrogen receptor-β (ERβ), whereafter the original estrogen receptor has been referred to as estrogen receptor-α (ERα) (Kuiper et al., 1996). Cloning of ERβ has open new possibilities for understanding and explaining certain cell type specific differences in estrogen responses. ERα and ERβ are members of the nuclear hormone receptor

superfamily that includes receptors for steroid and thyroid hormones, vitamin D_3 and retinoic acid. ERs consist of an A/B domain (N terminus), a DNA binding domain, a hinge region and a domain responsible for ligand binding, dimerisation and ligand-dependent transactivation function. ERα and ERβ are 96% homologous in the DNA binding domain and 53% homologous in the ligand binding domain, whereas the other domains show a lesser degree of homology. The main difference between the two receptor subtypes lies in the N terminal region, where only 16% homology is shown.

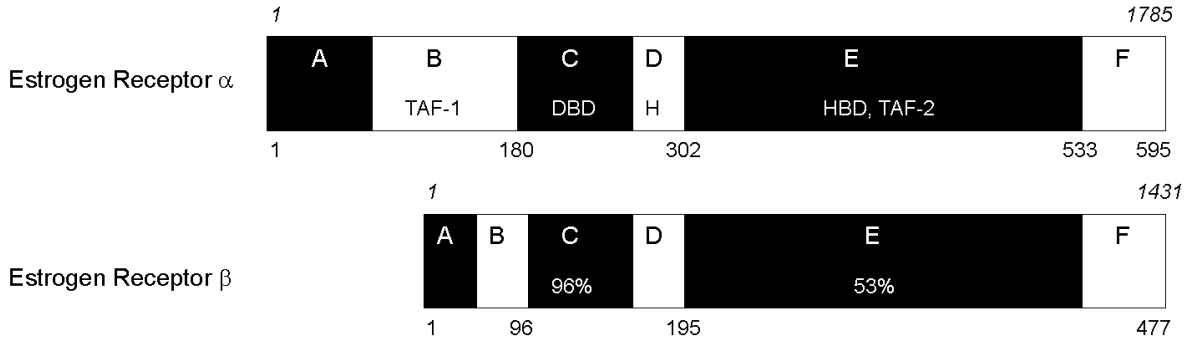


Figure 1: Structures of the estrogen receptors α and β

Estrogen receptors α and β are members of the steroid hormone receptor family and are organised into a similar domain structure as other members of this family. The amino-terminal A/B domains contain a ligand-independent transcriptional activation function (TAF-1). The DNA-binding domain (DBD) contains two zinc-fingers that are highly conserved and domain D contains the hinge region (H) of the ERs. The hormone-binding domain (HBD) and the hormone-dependent transcriptional activation function (TAF-2) is contained in domain E and domain F is a variable region including a sequence that might be important for the maintaining a specific conformational structure after ligand-binding. It is proposed that this region is important for the difference in response of estrogen receptors to estradiol and selective estrogen-receptor modulators (Brzozowski et al., 1997). Estrogen receptors α and β are 96% homologous in the DBD and 53% homologous in the HBD containing TAF-2, but have unique A/B domains. The numbers in italics above indicate the nucleotides and the numbers below show the amino acids.

1.3.1 Classical genomic mechanisms of gene induction

Ligand binding results in a conformational change in the ER, leading dissociation from heat shock protein cofactors that retain the ER in the cytosol and a subsequent translocation to the nucleus. In the nucleus, the ligand-bound ER binds to specific DNA sequences situated

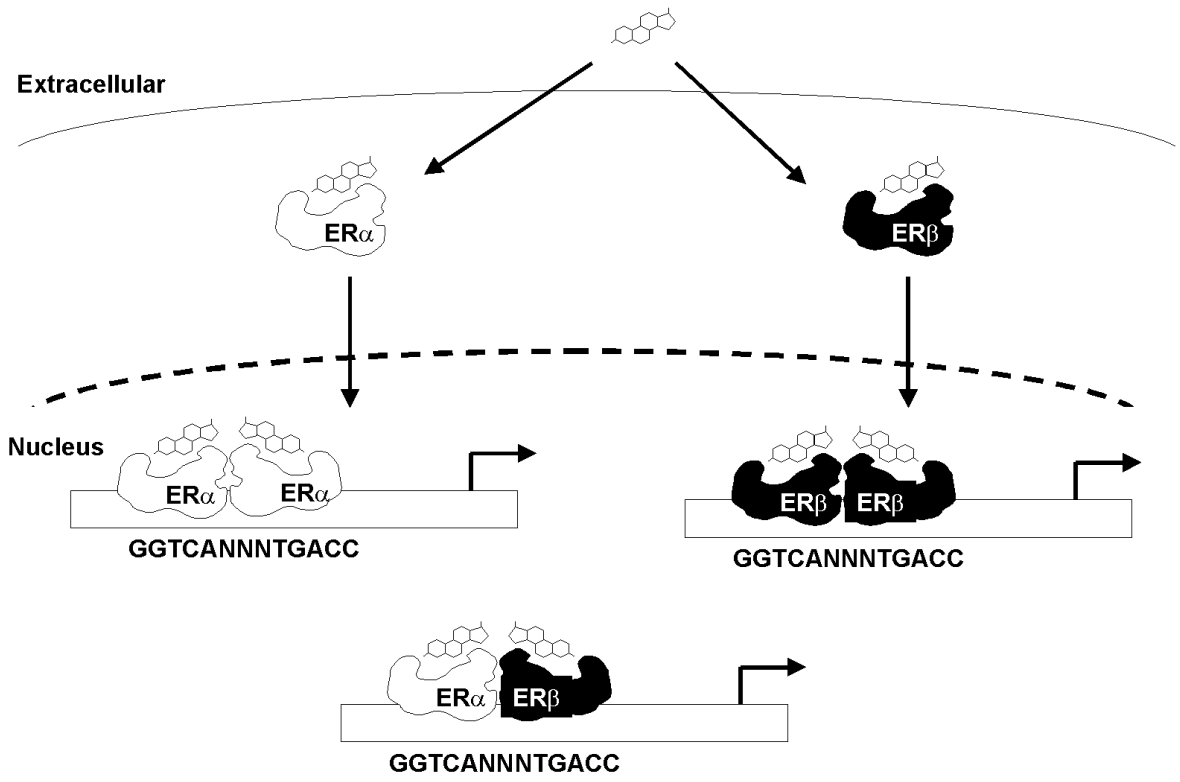


Figure 2: Classical mechanism of genomic estrogen receptor-dependent gene activation

Estradiol, when entering the cell and binds with high affinity to estrogen receptors - α and -β. ERα and ERβ undergo conformational changes upon estradiol binding which enables them to translocate to the cell nucleus. Estrogen-estrogen receptors complexes can either form ERα or ERβ homodimers which then bind to specific estrogen response elements (EREs) in the promoter regions of target genes. ERα and ERβ can also form heterodimers at the EREs, leading to modulation of target gene expression.

in promoter regions of target genes, the estrogen response elements (ERE). DNA-binding occurs via two zinc finger domains situated in the DNA-binding domain of both ERα and ERβ. EREs are palindromic sequences consisting of two 5 bp arms separated by a 3 bp spacer, with the classical consensus sequence being GGTCANNNTGACC. Ligand-bound ERs interact with palindromic EREs as homo- or heterodimers. Not only perfect palindromic EREs can confer an estrogen response to a promoter, but also a half-palindromic ERE. The chicken ovalbumin gene contains only one ERE half site present just upstream of the TATA box (Kato et al., 1992). A point mutation in this ERE ablates the estrogen response, demonstrating that this ERE half site is functional and confers an estrogen response to the

chicken ovalbumin gene. *In vitro* binding studies have however shown that ERs are unable to form a stable complex at this ERE half site, suggesting the need for specific co-factors that stabilises ER binding to ERE half sites. Once bound to the ERE, the hormone-receptor complex activates transcription of estrogen-responsive genes, which is mediated via transcriptional activation functions situated in the A/B and DNA binding regions of ERs.

1.3.2 Rapid non-genomic mechanisms of gene induction

Not all estrogen effects are long term genomic effects and many recent investigations have focussed on so-called non-genomic estrogen effects. Estrogen-treatment of various cell types can lead to the rapid activation of immediate early genes such as c-fos (Weisz et al., 1990). The induction of immediate early genes occurs within 5 to 15 minutes of estrogen-treatment, which is not in line with the classical genomic mechanism of gene activation. Recent work has shown that estrogen-treatment leads to rapid activation of the extracellular signal-regulated kinase (ERK1/2) within 5 minutes. Similar results were reported for the c-Jun-NH_2-terminal protein kinase (JNK) and the p38 MAPK in various cell types including human umbilical vascular cells (HUVECS), vascular smooth muscle cells, cardiac fibroblast as well as cardiomyocytes (Lee and Eghbali-Webb, 1998; Nuedling et al., 1999). This activation is estrogen receptor-dependent and results from a cross-talk between the estrogen receptor pathway and the signal transduction pathways of the cell. Investigations into the cytosolic upstream events have shown, that the estrogen receptor can directly interact with c-src in MCF-7 breast cancer cells (Migliaccio et al., 1996; Migliaccio et al., 1998), a protein closely associated with the cell membrane and one of the first proteins to be activated after a cell receives an external mitogenic signal. This direct interaction between the estrogen-receptor and c-src leads to activation of c-src, which is then capable of transmitting the signal to the rest of the signalling cascade, ultimately leading to gene regulation in the nucleus.

Another mechanism of rapid activation of the signalling cascade involves a rapid increase of second messengers such as cAMP and a rapid release of intracellular calcium upon

estrogen-treatment in a variety of cell types (Improta-Brears et al., 1999). Calcium and cAMP serve as second messengers responsible for the activation of various signalling pathways, leading to changes in gene expression necessary for regulating cellular functions such as cell proliferation. It has been shown that estrogen-treatment of MCF-7 breast cancer cells as well as vascular smooth muscle cells results in cell proliferation (Farhat et al., 1996). The "nuclear" response to activation of cell signal transduction pathways is the activation and recruitment of transcription factors responsible for gene regulation. Rapid "non-genomic" estrogen effects should therefore rather be seen as primarily "non-nuclear" effects finally resulting in a rapid nuclear response. Rapid non-nuclear estrogen receptor-dependent mechanisms of action therefore extents the effects of estrogen beyond genes containing classical EREs in promoter regions to genes regulated via other transcription factors activated upon estrogen-treatment. Therefore a need exists to understand the nuclear events, i.e. gene expression patterns and mechanisms of gene regulation, following cell signalling activation upon estrogen stimulation. Not much is known about the nuclear events (excluding the classical genomic estrogen actions) following estrogen stimulus and extensive investigations into transcription factor recruitment upon estrogen treatment is pivotal in getting a complete picture of the role estrogen receptors plays in regulating various cellular functions.

1.3.2.1 ERα, ERβ or a membrane estrogen receptor?

Since ERα and ERβ are classically thought of as "nuclear" receptors, the question into the mechanism of rapid non-nuclear effects of estrogen arose and whether the classical nuclear receptors or a novel, not yet identified membrane-associated estrogen receptor are mainly involved in rapid non-nuclear estrogen actions. Many studies, including studies performed in the uteri of ERα knock-out mice expressing very little amounts of ERβ (Das et al., 1997) have indicated the presence of a membrane estrogen receptor that is not ERα or ERβ (Nadal et al., 1998; Stefano et al., 2000). In fact, it has been suggested that non-genomic estrogen actions in pancreatic β-cells are mediated via a novel membrane receptor unrelated to ERα

and ERβ (Nadal et al., 2000), but this “novel” membrane receptor has not yet been cloned. On the other hand, various studies have indicated ERα as the receptor responsible for mediating rapid non-genomic estrogen effects (Kim et al., 1999, Chambliss, 2000; Norfleet et al., 2000; Schlegel et al., 1999) and a mechanism involving localisation of ERα in caveolae where it interacts with caveolin-1 (shown in NIH 3T3 fibroblasts) has been proposed as one plausible mechanism of rapid signal transduction upon estrogen-treatment (Schlegel et al., 1999). In immortalised pulmonary artery endothelial cells it has also been shown that ERα is localised to the caveolae. In this case ERα does not interact with caveolin-1 but with eNOS (endothelial nitric oxide synthase-1), proposing a mechanism for the rapid estrogen-induced NO release observed in endothelial cells (Chambliss et al., 2000). On the other hand it has been shown that when the classical nuclear ERα and ERβ are overexpressed in Chinese hamster ovary (CHO) cells, the receptors are mainly expressed in the nucleus and cytosol of the cells, but that a small amount of receptors are also associated with the cell membrane. Until a novel and definite membrane estrogen receptor is cloned, the debate on the involvement of the classical nuclear or a novel membrane estrogen receptor will continue. It is, of course, also possible that multiple mechanisms are working together to transmit rapid estrogen signals, and that both the classical nuclear receptors tightly associated with other membrane-proteins, as well as a novel, not yet isolated, membrane estrogen receptor are active and responsible for the non-genomic estrogenic actions.

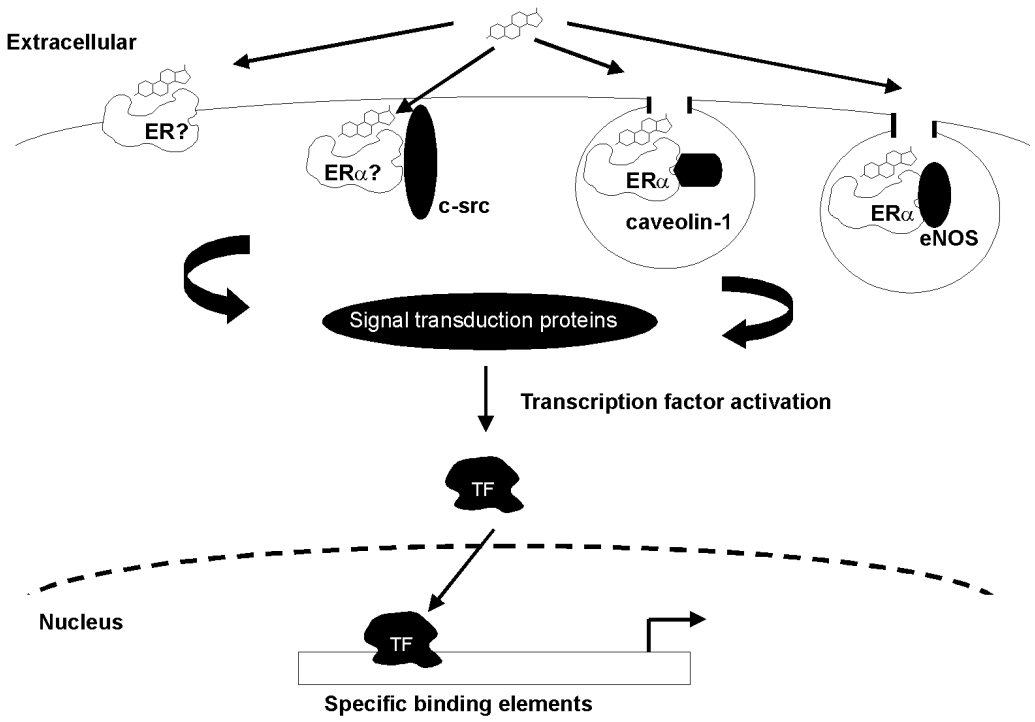


Figure 3: Rapid mechanisms of estrogen receptor action

Rapid activation of signal transduction pathways is currently postulated to be mediated via a cross-talk between the estrogen receptor (ER) pathway and the MAPK pathway. This is mediated by a direct interaction of ER with c-src, a membrane-associated protein responsible for the downstream activation of the MAPK pathway components. Evidence for a membrane-bound ER as well as ERs in caveolae that interact with either caveolin-1 or eNOS has also been provided. Rapid activation of the signal transduction machinery of the cell leads to the activation of numerous transcription factors that translocate to the nucleus where it binds to specific binding sites in promoter regions of target genes.

1.4 Estrogen and estrogen receptors in the myocardium

Estrogen receptors have been identified in vascular smooth muscle cells, vascular endothelial cells, cardiac fibroblasts as well as cardiomyocytes. In the myocardium estrogen receptors are functional and can drive the expression of estrogen target genes such as connexin 43 (Yu et al., 1994), L-type calcium channel (Johnson et al., 1997; Patterson et al., 1998), c-fos (Grohe et al., 1996) and Egr-1 (De Jager et al., 2001). A protective role for estrogen has been attributed to the vascular system, but if this protective role can also be extrapolated to the myocardium itself is not yet clear and needs further extensive investigation. Clinical studies and hormone replacement investigations have so far mainly focussed on vascular disease such as atherosclerosis and investigations of the role of

estrogen in myocardial disease such as hypertrophy and heart failure has been largely neglected in clinical trials. One clinical study, however, reported a beneficial effect of hormone replacement therapy on cardiac hypertrophy, where a lower rate of left ventricular hypertrophy was found in 574 postmenopausal women with combined estrogen and progesterone HRT (Kuch et al., 1997). Interpretation of the results of this study were however complicated by the significant demographic differences between the groups. Another small study with 39 normotensive postmenopausal women identified an association between long-term HRT and lower left ventricular weight compared to women not receiving HRT (Lim et al., 1999). These two studies therefore indicated, but did not prove, that estrogen might play a role in the prevention of primary cardiac hypertrophy. The role of estrogen in the treatment of cardiac hypertrophy in cases where the disease is already established is still very controversial, and the results of the HERS study indicate that estrogen-treatment has no effect on treating cardiac hypertrophy after onset (Herrington, 1999). Estrogen therefore, should probably be seen as playing a protective role in preventing primary cardiac disease, rather as a potential treatment in cases where cardiac disease is already established.

In gonadectomised animals, estrogen replacement restored ventricular function in a working heart model (Scheuer et al., 1987). This was not the case for progesterone substitution, indicating that the beneficial effects were due to estrogen and not to progesterone contained in the HRT. Furthermore in this study, estrogen also blocked the hypertrophy induced shift in myosin isoenzyme expression towards more β-MHC, which was not blocked by progesterone, again indicating specific beneficial effects of estrogen on myocardial function. Interestingly testosterone had similar beneficial effects than estrogen on myocardial function in both male and female rats (Scheuer et al., 1987), which might be explained by active aromatase identified in cardiomyocytes that is metabolising local testosterone to estrogen in the cardiomyocytes themselves. Previously our group has shown that estrogen protects cardiac myocytes by an inhibition of myocyte apoptosis (Pelzer et al., 2000). In the failing

human myocardium, apoptosis has been identified as one of the mechanisms responsible for myocardial cell loss. In isolated cardiomyocytes estrogen inhibited the staurosporine-induced activation of caspase-3, one of the central proteases responsible for the initiation of the caspase-cascade ultimately leading to cell death. This inhibition of apoptosis was specifically mediated via the estrogen receptors, since it was blocked by a specific estrogen receptor antagonist ICI 182780. Therefore, this data indicated that estrogen, acting through the estrogen receptors, could be involved in various mechanisms of myocardial protection, one of which might be by an inhibition of apoptosis. However, the anti-apoptotic effects of estrogen are probably not the only mechanisms by which estrogen exerts a positive effect on the myocardium. To date there is still a lack of complete and detailed knowledge concerning the role, function and mechanisms of action of estrogen and estrogen receptors in the myocardial cells themselves. Clinical studies extended to myocardial disease as well as research directing the attention to cardiomyocytes in stead of vascular cells will help in understanding the overall picture of estrogen action in the heart.

1.5 Hormone replacement therapy as treatment for cardiovascular disease?

Various studies have repeatedly described a beneficial effect of estrogen on the lipid profile in postmenopausal women receiving HRT (Davis et al., 1994; Kannel et al., 1976; Matthews et al., 1989). As early as the 1950s, HRT has been proposed as potential therapy in the treatment of cardiovascular disease due to the reduction of lipid levels by estrogen (Barr et al., 1952). After accounting for lipid effects, significant beneficial effects of HRT remains (Bush et al., 1987) and alternate cardiac or vascular effects of HRT have been sought. Receptors for estrogen, progesterone and androgen have been identified in cardiac (Grohe et al., 1997,Marsh et al., 1998; Weinberg et al., 1999) and vascular tissue (Karas et al., 1995; Mashiah et al., 1999), supporting the hypothesis that female sex hormones play a major role in the modulation of cardiovascular function. The main focus of most HRT studies have, however been the role in coronary artery disease, thereby mostly ignoring direct myocardial effects. A large range of non-lipid related beneficial effects in vascular function has been

described. These include an alteration in the fibrinolytic balance (Koh et al., 1997; Shahar et al., 1996), induction of prostacyclin (Chang et al., 1980), improvement of endothelial function (Chang et al., 1980; Gilligan et al., 1994; Gisclard et al., 1988; Keaney et al., 1994; Lieberman et al., 1994; McCrohon et al., 1996; White et al., 1997), NO release (Collins et al., 1995; Hayashi et al., 1992; Weiner et al., 1994), attenuation of endothelin effect (Jiang et al., 1992) and calcium blockade (Collins et al., 1993; Freay et al., 1997; Jiang et al., 1992; Weiner et al., 1994). Furthermore direct effects on vascular depolarisation (Harder and Coulson, 1979) and smooth muscle relaxation (Magness and Rosenfeld, 1989; Mugge et al., 1993) have been described. One common outcome of all these mechanisms explaining the beneficial effects of estrogen involves vasodilatation, which was associated with increased cardiac output (Pines et al., 1991; Slater et al., 1986). The use of pure estrogen may also have severe negative effects in postmenopausal women, with increases in the risk of breast cancer as well as an increased risk of venous thrombosis (Grodstein et al., 1996).

Novel strategies are currently being developed where the beneficial effects of estrogen on the cardiovascular system can be gained without the numerous side-effects associated with HRT. Alternate drugs for treatment of cardiovascular disease in men will also be advantageous. Novel classes of selective estrogen-receptor modulators (SERMs) have been developed, which have different effects in regulating gene expression depending on the tissue and whether they are bound to ERα or ERβ (Mitlak and Cohen, 1997). These compounds impart different structures to the ligand-binding domain of ERα (Kuiper et al., 1997), when bound to the receptor compared to the structure of the receptor when bound to the natural ligand estrogen which might explain the tissue-specific effects of these compounds. It has for example been shown, that raloxifene favourably alters several cardiovascular risk markers in healthy postmenopausal women, exerting the potential to decrease cardiovascular risk (Cummings et al., 1999). Tamoxifen is also a compound which has been associated with a reduced risk of coronary artery disease.

The roles of ERα and ERβ have not completely been clarified. Differences in modulating gene expression by ERα and ERβ via AP-1 sites have been described, proposing different effects of the two receptors on gene expression patterns (Paech et al., 1997). Further evidence that the two receptors have different functions comes from ERα knock-out animals. ERα knock-out mice are infertile despite the presence of ERβ (Korach et al., 1996), whereas the ERβ knock-out mice are fertile, suggesting a role for ERα in the reproductive system. ERβ seems to play important roles in the brain and vascular system, strongly favouring specific ERα and ERβ modulators for developing tissue-based treatment possibilities. Currently ERα and ERβ-specific agonists have been developed and are being tested with respect to the effects on myocardial gene expression and function. It will be interesting and important to clarify the roles of the two receptors in the myocardium, but also in other tissues to determine whether these substances might prove to be useful in the treatment of cardiac disease with reduced side effects in other tissues.

1.6 Cardiac hypertrophy

High blood pressure (hypertension) is the largest single risk factor for the development of heart failure in western countries (Levy et al., 1996). Sustained hypertension imposes an excessive workload on the heart causing the heart to respond to prolonged stress by the development of hypertrophy which is characterised by an increase in myocyte size and subsequent thickening of the ventricle wall. Initially the development of cardiac hypertrophy is an adaptive mechanism to retain normal cardiac function in response to a variety of mechanical, hemodynamic, hormonal and pathological stimuli causing the heart to adapt to the increased demands on cardiac output (Opie, 1998). These compensatory mechanisms are, however, short term protective mechanisms with longer term deleterious effects. Adaptive changes of the heart occur at cellular level, with changes in the morphology and phenotype of the cardiac myocytes. So-called physiological hypertrophy occur for example in athletes and is associated with proportional increases in the length and width of the cardiac myocytes. This form of hypertrophy is not associated with changes in the gene expression

pattern observed with volume or pressure overload and does usually not progress to heart failure. During sustained pressure overload, such as in long term hypertension, concentric hypertrophy occurs which is characterised by an increase mainly in the width of the myocytes. These morphological changes in the cardiac myocytes cause the ventricular wall to thicken, which again reduces the stress on the wall due to pressure overload according to the law of LaPlace.

In order for the myocytes to adapt to increased pressure overload by phenotypical changes, physical mechanical forces must be transduced into biochemical signals leading to changes in myocardial gene expression. Studies in isolated cardiac myocytes identified a multitude of growth factors, hormones and cytokines that result in a hypertrophic answer. Similarly a variety of signalling mechanisms have been identified as the key role players in the transmission of a hypertrophic stimulus. Various hypertrophic stimuli and signalling pathways have been identified using isolated cardiomyocytes as well transgenic and knock-out animals and one common feature of all stimuli and signalling pathways is the re-expression of a fetal gene program. Among the changes in gene expression pattern is a so-called switch from predominantly α-myosin heavy chain (MHC) in the adult rodent heart to predominantly β-MHC (Stephens and Swynghedauw, 1990). Other changes in gene expression include upregulation of α-skeletal muscle actin and a downregulation of α-cardiac actin, upregulation of atrial myosin light chain-1 and a downregulation of ventricular myosin light chain-1, upregulation of atrial natriuretic peptide (ANP) and a downregulation of brain natriuretic peptide (BNP) and downregulation membrane proteins such as SERCA2.

1.6.1 α- and β- Myosin heavy chain expression

The switch in the expression from mainly α-MHC to predominantly β-MHC has traditionally been used as a marker for cardiac hypertrophy, at least in rodents (Schwartz et al., 1992; Stephens and Swynghedauw, 1990). Myosin heavy chain is one of the major contractile proteins in the heart and exists as two isoforms. α-MHC is mainly expressed in the adult

heart of small rodents, whereas β-MHC is predominantly expressed in the fetal and neonatal heart. The MHCs consist of a N-terminal globular head region and a C-terminal rodlike α-helical tail region. In native myosin, two α-helical rod segments of two myosin heavy chains are coiled around each other to form a long rigid coiled-coil structure with 2 globular heads to which the myosin light chains 1 and 2 are bound. In muscle, the coiled-coil tails of 300 – 400 myosin dimers are packed together to form the thick filament. The head regions of the MHC contains an actin-binding site as well an ATP binding site which are essential for muscle contraction. Binding of ATP to the ATP binding site in the head region of the MHCs and subsequent hydrolysis of ATP to ADP serves as the driving force that powers muscle contraction. The velocity of contraction is dependent on the speed of ATP hydrolysis, i.e. the ATPase activity of the MHCs situated in the head region. α-MHC has been shown to contain a three times higher ATPase activity than β-MHC (Weiss and Leinwand, 1996). Small animals such as mice and rats have predominantly α-MHC and also a very fast heart rate, whereas bigger animals and humans have predominantly β-MHC and also a much slower heart rate. It has until recently been believed that only β-MHC is expressed in the human heart, but improved electrophoretic techniques to separate the 2 large proteins with a 93% homology have made it possible to also identify α-MHC in the human heart. In hearts of patients with heart failure, α-MHC is downregulated to the extent that it is not detectable any more, making the downregulation of α-MHC observed in rodents also applicable to humans (Lowes et al., 1997; Miyata et al., 2000; Nakao et al., 1997).

1.6.2 The spontaneously hypertensive rat as a model for cardiac hypertrophy

The spontaneously hypertensive rat (SHR) is a well established model for hypertensive heart disease in humans. The animals exhibit compensated cardiac hypertrophy for more than 50% of their lifespan, becoming susceptible to cardiac dysfunction and congestive heart failure at approximately 21 months of age (Bing et al., 1995; Pfeffer et al., 1979). The primary stimulus for hypertrophy and failure in these animals is hypertension. Phenotypically SHR animals resembles human cardiac hypertrophy with respect to elevated systolic blood

pressure, compensated left ventricular hypertrophy, progressive increase in left ventricular volume and fibrosis (Engelmann et al., 1987; Pfeffer et al., 1979). These phenotypic characteristics are present from early adulthood throughout the lifespan of the animals and at approximately 18 months of age, SHR begin to develop signs of heart failure associated with depressed myocardial function, extensive interstitial fibrosis and apoptotic myocyte loss (Bing et al., 1995; Conrad et al., 1995; Li et al., 1997). Changes in gene expression observed in other models of cardiac hypertrophy such as an α-/β-MHC switch, increased ANP expression and downregulation of SERCA2 are observed in the hearts of SHR animals. These animals were therefore the model chosen in the present work for investigating the effects of estrogen on the myocardium in the setting of cardiac hypertrophy.

2 Aims of the study

The aim of the study was to investigate the role and mechanisms of estrogen action in the myocardium. This was investigated *in vivo* in an animal model for cardiac hypertrophy as well as *in vitro* in isolated neonatal cardiac myocytes. The following questions were addressed during the course of the study:

1) Does estrogen, acting via the estrogen receptors, directly affect myocardial gene expression in vivo? This question was addressed by an *in vivo* analysis of the effects of estrogen on the expression of one of the major cardiac specific contractile proteins, the α-myosin heavy chain.

2) What are the mechanisms of action of estrogen receptors in regulating the expression of myocardial genes? This question was partly answered by investigating the mechanisms involved in the estrogen-induced rapid upregulation of the early growth response gene-1 (Egr-1), an estrogen-target gene in the myocardium previously identified by our group. Attention was given to the promoter elements conferring an estrogen response to Egr-1 in isolated cardiomyocytes, as well as to the recruitment of transcription factors by estrogen leading to gene activation.

3) What is the spectrum of genes that are modified by estrogen in the myocardium? To start to address this question, a cDNA array approach was initiated to identify potential novel myocardial estrogen target genes, in order to obtain a better picture of the global effects that estrogen exerts in the myocardium.

3 Methods

3.1 *In vivo* animal study

Six week old female spontaneously hypertensive rats (SHR) were ovarectomised or sham operated, followed by daily injections of peanut oil alone (control) or peanut oil containing 2 µg/kg body weight 17β-estradiol alone or in combination with 250 µg/kg body weight estrogen receptor antagonist ICI 182780. The animals were treated over a period of 12 weeks and at 18 weeks of age systolic blood pressure was measured by tail cuff before the animals were sacrificed by decapitation. Organs were removed, shock frozen and stored at –80°C for analyses. The serum estrogen levels of all animals were determined by standard radio immunoassay (RIA; DPC Bierman).

3.2 *In vitro* analyses in isolated primary neonatal cardiomyocytes

3.2.1 Preparation of primary neonatal cardiomyocytes and cell culture

Primary neonatal cardiomyocytes were prepared from the hearts of 2 - 3 day old Wistar rats. The thorax was disinfected by an iodine solution, the animals decapitated and the thorax opened with a pair of scissors until the hearts were exposed. Hearts were carefully removed and collected in CBFHH buffer containing 1 ml Heparin to prevent the blood from clotting. After all hearts were collected, they were washed with fresh CBFHH buffer and cut into small pieces with a sterile scalpel. Heart pieces were transferred to a 50 ml Falcon tube containing a sterile magnetic stirrer bar and digested in approximately 15 ml CBFHH buffer containing trypsin and DNAse I (T&D). Digestion were performed for 20 minutes by continuous mixing on a magnetic stirrer. After the first 20 minute digestion, the heart pieces were mixed by careful up - and down pipetting, allowed to settle at the bottom of the tube and the supernatant removed and discarded. This step was repeated, again discarding the supernatant. Hereafter, the digestion steps were performed for 5 minutes, each time mixing the heart pieces in T&D well, and collecting the supernatant in 50 ml Falcon tubes containing 7.5 ml charcaol stripped FCS to inhibit the trypsin. This procedure was continued until all the heart pieces were completely digested away. Cells were pelleted by centrifugation at 1200

rpm for 5 minutes and resuspended in MEM/5. Preplating was performed by running the cell mixture through a cell strainer to remove all bigger undigested pieces and incubating the plated cells at 37°C / 1% CO_2 for 2 hours. This step allows for the removal of contaminating fibroblast, since fibroblast settle at the bottom of the Petri dish within 1 to 2 hours after plating leaving the cardiomyocytes in the supernatant. After pre-plating, the cardiomyocytes contained in the supernatant were removed, taking care not to wash the fibroblast off the bottom of the Petri dishes. Cardiomyocytes were collected and counted in a Fuchs-Rosenthal chamber by the addition of Trypan-blue to dissociate between viable and non-viable cells. Cells were subsequently plated at a density of 25 000 - 35 000 cells per cm^2. The next day, medium was changed to MEM containing 2 % CPSR1. All experiments except the transfection experiments were performed in MEM without serum or CPSR1 ("hunger medium"). Medium was changed to "hunger medium" one day before the experiments were performed.

3.2.2 RNA preparation

Total RNA was isolated from cultured cells as well as from tissues using the Trizol reagent according to manufacturer's protocols. RNA quality was analysed by electrophoresis in formaldehyde containing 1 % agarose gels using a MOPS buffer. The integrity of the 18 S and 28 S rRNA bands were an indication of RNA quality.

3.2.3 RNase Protection Assay

The pBS-MHC riboprobe was linearised with Bam HI, electrophoresed in a 1% agarose gel and extracted from the gel slice using standard methods. The following *in vitro* transcription reaction was set up:

4 µl 5 X Transcription buffer

1 µl 100 mM DTT

1 µl Rnasin (Promega)

3 µl CTP, ATP, GTP mixture (3.3 mM each)

1 µl 0.5 mM UTP

350 ng pBS-MHC riboprobe

250 ng pTri-28S antisense probe

5 µl ^{32}PαUTP (10 mCi/µl)

1 µl T_7 RNA Polymerase (10 U/µl)

The reaction was incubated at 37°C for 30 minutes, before the plasmid DNA was removed by the addition of 30 µl 1 X transcription buffer, 1 µl RNasin and 10 units RNase free DNase I and incubated at 37°C for 15 minutes. Free radionucleotides and salts were removed by cleaning up the reaction mixture over a RNase free Sephadex G 50 column. 1 X 10^5 cpm of RNA probe was hybridised to 10 µg of total RNA in 1 X PIPES buffer and 50% deionised formamide. Prior to hybridisation, the hybridisation mixture was denatured at 85°C for 15 minutes and immediately transferred to a hybridisation oven at 55°C. Hybridisation was performed overnight to allow the formation of double stranded RNA hybrids between the sample RNA and the radioactively labelled RNA probe. After hybridisation, the single stranded RNA was digested by adding 300 µl of an RNase cocktail (10 minutes incubation at 37°C). 20 µl 10 % SDS and 5 µl Proteinase K (10 mg/ml) was added to inactivate RNases and incubated at 37°C for 15 minutes, whereafter a phenol/chloroform extraction was performed to remove SDS and Proteinase K. The RNA-hybrids were precipitated with cold 100% ethanol in the presence of 2 µg yeast tRNA at -20°C for 1 hour. The RNA-hybrids were pelleted by centrifugation, washed with cold 80% ethanol and dissolved in 5 µl of loading dye. Samples were denatured at 93°C for 2 minutes before they were resolved in 6% denaturing poly-acrylamide gels that were pre-run for 1 – 2 hours at 500 V in 0.5 X TBE buffer. Gels were dried under vacuum at 80°C for 45 minutes and exposed to X-ray film and phosphoimager for analyses.

3.2.4 Protein methods

3.2.4.1 Antibodies used

Primary antibody	Application	Size	Dilution	Ig	Company
α-MHC	WB	250 kDa	1:3000-1:5000	Mouse IgG1	Biocytex biotechnology #1170-S
β-MHC	WB	250 kDa	1:3000-1:5000	Mouse IgG2a	Biocytex biotechnology #1040-S
Egr-1	WB/EMSA	85 kDa	1:500	rabbit polyclonal	Santa Cruz #sc-189
ERK1/2	WB	44 and 42 kDa	1:500	rabbit polyclonal	New England Biolabs #9102
phospho-ERK1/2	WB	≈ 50 kDa	1:500	rabbit polyclonal	New England Bioloabs # 9101S
p38 MAPK	WB	38 kDa	1:1000	rabbit polyclonal	New England Biolabs #9212
phospho-p38 MAPK	WB	≈ 40 kDa	1:500	rabbit polyclonal	New England Biolabs #9211S
SRF	EMSA	-	1 µg/EMSA	rabbit polyclonal	Santa Cruz #sc-335

3.2.4.2 Protein extraction for Western blotting

Proteins were extracted from tissue or cultured cells by lysis of the cells in RIPA buffer or homogenisation of tissue in RIPA buffer. The cell lysates or tissue homogenates were centrifuged at 15 000 rpm for 15 min at 4°C to remove nuclei and the insoluble fraction and stored at –80°C. Protein concentration was determined by the Bradford protein assay using a BSA standard curve.

3.2.4.3 Western blotting

Depending on the size of the target protein, proteins were separated on 7.5 % - 15 % SDS-Glycine polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes following the manufacturer's recommendations. After transfer, the membranes were washed in PBS-T to remove traces of methanol and SDS and dried on Whattman filter paper to fix the proteins on the membranes. Membranes were blocked in 5% non-fat milk powder in PBS-T for at least 1 hour at RT, followed by incubation with the specific primary antibody diluted in PBS-T containing 2% non-fat milk powder at RT for at least 1 hour. After incubation with primary antibody, the membranes were washed 5 times with large volumes of PBS-T and incubated with a horseradish peroxidase labelled secondary antibody for 45 minutes at RT. The secondary antibody was removed, membranes washed with large volumes of PBS-T and the protein detection performed using the enhanced chemiluminescence (ECL) according to the manufacturer's recommendations.

3.2.4.4 Protein preparation for α - MHC gel electrophoresis

Protein for α-MHC gel electrophoresis was prepared from frozen ventricle tissue. 20 mg of tissue was homogenised in 200 µl of α-MHC sample buffer containing a proteinase inhibitor cocktail. After homogenisation the samples were centrifuged at 15000 rpm for 15 min at 4°C to remove the nuclei and insoluble protein fraction and the supernatant was stored at –80°C in small aliquots. Protein concentration was determined in duplicate by the Bradford protein assay using a BSA standard curve.

3.2.4.5 α - MHC gel electrophoresis

Protein samples were fractionated in 6% SDS and glycerol containing polyacrylamide separating gels with 4% collecting gels. Electrophoreses were performed at 200 V for 18 hours followed by silver staining of the gels and densitometric analysis by the Scanpack software package.

3.2.5 Plasmid DNA

A list and map of all plasmids used is contained in Appendix I.

Plasmid DNA was amplified in DH5α strain *Epicurian coli (E.coli)* bacterial cells that were made competent by the standard $CaCl_2$ – method. 100 ng of plasmid DNA was incubated with 50 µl competent bacterial cells for 30 minutes. The bacterial cells were heat shocked at 42°C for 30 seconds, 500 µl LB medium (Appendix I) was added and cells were grown at 37°C for 1 hour in a shaking incubator to allow for the expression of the antibiotic resistance genes. Aliquots were then spread onto LB –agar plates containing the desired antibiotic and cells were grown overnight at 37°C. A single colony of antibiotic resistant bacterial cells were inoculated into 200 ml LB medium containing the appropriate antibiotic, grown overnight and plasmid DNA was isolated according to standard methods (Macheray and Nagel Maxi Prep kit).

3.2.6 Plasmid construction: cloning of α-MHC promoter in front of luciferase reporter

The 5.4 kb mouse α-MHC promoter was cloned into the SacI site of the pGL3-basic luciferase vector (Promega). Plasmid DNA was digested with Sac I in the appropriate buffer as recommended by the manufacturer. DNA was separated on 1% agarose gels (Sigma) in 1XTAE buffer and the desired fragments were cut out of the gel with a clean scalpel. DNA fragments were extracted from the agarose gel pieces using the Qiaquick gel extraction system (Qiagen). Linearised vector was dephosphorylated at 37°C for 30 minutes using a shrimp alkaline phosphatase (Roche) followed by inactivation of the enzyme at 65°C for 45 minutes. The insert and vector were ligated using T4-DNA ligase (Stratagene) at 4°C overnight, followed by transformation into XL10 Gold supercompetent *E. coli* cells as described.

3.2.7 Transfection of primary cardiomyocytes using the poly-L-lysine component system

Primary cardiomyocytes were transfected using an adenovirus-based carrier system for myocardial cells extensively modified by our group. $4x10^{10}$ p/mL replication deficient RR5

adenovirus were coated with poly-L-lysine for 50 minutes. (Note: a final concentration of 2 mg/ml poly-L-lysine was desired and two-thirds of the amount of poly-L-lysine was added during the first incubation step with virus). 1µg of DNA was added to the virus-poly-L-lysine mixture and incubated for 30 minutes to bind the DNA to the poly-L-lysine. Another coat of poly-L-lysine (here one-thirds of the total amount of poly-L-lysine is added) was added and the DNA-bound virus was incubated with the cells in serum- and antibiotic-free MEM:OptiMEM (4:1, Life Technologies) for 2.5 hours. After 2.5 hours virus particles in the transfection medium were removed by washing, fresh growth medium was added and incubated overnight. Luciferase activity was measured using a luciferase assay kit (Promega) and protein measurement served to standardise the transfections. Data is expressed as fold induction compared to untreated controls ± SEM for the indicated number of experiments.

3.2.8 Electrophoretic Mobility Shift Assay

3.2.8.1 Preparation of nuclear protein extracts from primary neonatal cardiomyocytes

Nuclear extracts were prepared from 1 X 10^7 cardiomyocytes. The cells were washed with PBS, carefully scraped off the Petri dishes in PBS and collected in a 1.5 ml centrifuge tube. The cells were carefully pelleted, resuspended in 400 µl buffer A and allowed to swell on ice for 15 minutes before lysis by forcing the cells 8 times through an insulin syringe. The nuclei were pelleted by centrifugation at 5 000 g for 5 minutes at 4°C. The supernatant was either discarded or used for the isolation of cytosolic proteins. The nuclear pellet was resuspended in 50 µl Buffer C. Samples were incubated on ice for 15 to 20 minutes, vortexing every few minutes, followed by centrifugation at 13 000 g for 5 minutes at 4°C. The supernatant containing the isolated nuclear proteins was stored at –80°C.

3.2.8.2 EMSA

For each EMSA 3 µg of nuclear protein extracts were incubated with 100 000 cpm of a ^{32}P-labelled oligonucleotide in 1 X binding buffer in the presence of 1 µg poly-dI/dC per 3 µg of

protein. Where indicated, 1 µg of an unlabelled specific or unspecific competitive oligonucleotide was added to the reaction mixture. Samples were incubated at 4°C for 10 minutes before loading onto 5% polyacrylamide gels that were pre-run for 2 hours in 0.5 X TBE buffer. Electrophoresis was continued at 220 V to fractionate the protein-DNA complexes. Gels were removed from the glass plates with 3MM Whattman paper, dried under vacuum at 80°C and exposed to X-ray film. For supershift assays, 1 µg of antibody was added to the reaction mixture. The oligonucleotide probe for SRE5 EMSA was as follow: 5 ' – AAACGCCATTATAAGGAGCAG – 3' . The underlined nucleotides represent the SRE5 in the Egr-1 promoter.

3.3 cDNA Array analyses

Total RNA was prepared from the left ventricles of ovarectomised animals as well as ovarectomised animals treated with 17β-estradiol as described in section 3.2.2. RNA quality was checked on a 1% formaldehyde-containing agarose gel and RNA from 5 animals per group were pooled for the cDNA array analyses.

3.3.1 DNAse treatment of total RNA

RNA was controlled for DNA contamination by PCR using primers for the rat GAPDH gene (see Appendix for primers sequences and PCR conditions). If a DNA contamination was detected, RNA was treated with RNAse-free DNAse I. 50 µg of RNA was treated with DNAse I in the following reaction: 50 µl RNA (1µg/µl)

10 µl 10 x DNAse I Buffer

5 µl DNAse I (1 u/µl)

35 µl sterile H_2O

The reaction was incubated at 37°C for 1 hour, 10 µl 10 X termination mix (0.1 M EDTA, 1 mg/ml glycogen) was added and extracted with 100 µl phenol:chloroform:isoamyl alcohol (25:24:1). The mixture was vortexed thoroughly and centrifuged at 14 000 rpm for 10 min to separate the phases. The aqueous phase was carefully removed to a clean 1.5 ml

microcentrifuge tube with an RNAse-free pipette tip and the RNA precipitated by the addition of 10 µl 7.5 M NH_4OAc and 150 µl 96% ethanol. The mixture was thoroughly vortexed and centrifuged at 14 000 rpm for 20 minutes. The supernatant was carefully removed and discarded and the pellet was washed with 100 µl 80% ethanol by centrifugation at 14 000 rpm for 10 minutes. The ethanol was removed, taking care not to loose the pellet, the pellet was air-dried and dissolved in 10 µl sterile H_2O. RNA is now at a concentration of 5 µg/µl.

3.3.2 First strand cDNA synthesis from total RNA

5 µg of total RNA was converted to ^{32}P-labeled first-strand cDNA according to the following protocol: 1µl of DNAse I-treated RNA (5 µl/µl) was combined with 1 µl of the 10 x CDS Primer mix in a 0.5 µl PCR tube. 1 µl H_2O was added to give a final volume of 3 µl. The mixture was incubated in a preheated PCR machine at 70°C for exactly 2 minutes. The samples were then quickly removed to a hybridisation oven preheated to 50°C without allowing the sampled to cool down and incubated at 50°C for 2 minutes. In the mean time, a reaction mixture containing the following reagents was prepared and 8 µl was added to the RNA and cDNA primer mixture:

2 µl 5 x Reaction buffer

1 µl 10 x dNTP mix for dATP labelling

3.5 µl α-^{32}P dATP (3 000 Ci/mmol, 10 mCi/µl)

0.5 µl 100 mM DTT

1µl MMLV reverse transcriptase (50 u/µl)

The contents was mixed well by carefully pipetting it up and down, without allowing the mixture to cool down. cDNA synthesis was continued at 50°C for 25 minutes. The reaction was stopped by adding 1 µl of a 10 X termination mixture.

Labelled cDNA was purified from unincorporated ^{32}P-dATP nucleotides over a Sephadex column provided by the company. Briefly, the columns were allowed to reach room temperature before use and the gel matrix completely suspended without introducing air

bubbles. The bottom and top caps were removed and the H_2O allowed to drain by gravity flow. The sample was carefully applied to the centre of the gel bed, 40 µl of H_2O was added and allowed to drain out of the column. 250 µl of H_2O was added and again allowed to drain and discarded. Now 4 fractions were collected each time adding 100 µl of H_2O, allowing it to drain and collected in a clean tube. The four fractions were then counted in a β-counter. Normally the 2nd and 3rd fractions contains the labelled cDNA and only these fractions are combined and used for hybridisation to the cDNA array filters.

3.3.3 cDNA Array membrane prehybridisation and hybridisation

Two cDNA array filters were hybridised in parallel, each with the labelled cDNAs to be compared. It is important to do the experiments in parallel, to be able to directly compare the results. Hybridisations were performed as follow:

15 ml of ExpressHyb was warmed at 68°C and 1.5 mg of denatured sonicated herring sperm DNA (10 mg/ml) was added. The filters were wetted with H_2O and each placed in a hybridisation bottle. 10 ml of hybridisation solution containing herring sperm DNA was added to the filter and prehybridised at 68°C for at least hour with continuos rotation. The cleaned-up cDNA was denatured by adding 20 µl of denaturing solution (1 M NaOH, 10 mM EDTA) and incubated at 69°C for 20 minutes. Thereafter 5 µl of C_ct-1 DNA (1µg/µl) and 200 µl neutralising solution (1 M NaH_2PO_4) was added and incubated at 68°C for 10 minutes. The whole mixture was then mixed with the remaining pre-warmed hybridisation solution containing herring sperm DNA (the final probe concentrations should be 0.5 to 2 X 10^6 cpm/ml) and the prehybridisation solution replaced by the hybridisation solution containing the labelled cDNA probe. Hybridisation was performed at 65°C overnight.

The next day, the hybridisation solution was removed and replaced with a big volume of 2 X SSC/ 1 % SDS. Filters were washed at 68°C for 30 minutes. This step was repeated 3 times. Two additional high stringency washes were performed using 0.1 X SSC/ 0.5% SDS at 68°C. The filters were carefully monitored with a Geiger-counter for background. If the background

signal was not too high, the filters were sealed in a plastic bag and exposed to a phosphoimager and X-ray film. Short, middle and long exposure times were chosen to be able to clearly detect genes that are highly expressed as well as those, that are less abundantly expressed.

3.3.4 Analyses

Analyses of phosphoimages as well as scans of X-ray films were performed by using the AtlasImage software according to the manufacturer's suggestions.

4 Results

4.1 *In vivo* regulation of α -myosin heavy chain expression by estrogen: long term animal study

4.1.1 Serum estrogen level and uterus weight of SHR animals

The serum estrogen levels of all the animals used in the study as well as the weight of the uteri were measured to control for the efficiency of ovarectomy and 17β-estradiol substitution. Ovarectomy resulted in an almost complete loss of serum estrogen (average of 0.523 ± 0.438 pg/mL compared to 27 ± 17.8 pg/mL of sham operated animals) as well as a drastic reduction of uterus size (average weight of 26.32 ± 1.7 mg/cm tibia length compared to 121.7 mg/cm tibia length of sham operated animals) (Figure 4a and b). Tibia length was chosen to standardise the uteri weight measurements. Normally body weight is used to normalise organ weight measurements for the size of the animals, but in this case, it was not possible to use body weight, since the animals treated with 17β-estradiol gained more weight during the 12 weeks of treatment than untreated animals.

17β-Estradiol substitution over a period of 3 months prevented the loss of serum estrogen levels as well as uterus atrophy caused by ovarectomy. An average serum estrogen level of 82.6 ± 13.7 pg/mL was reached by the substitution with 2 µg/kg 17β-estradiol. Although this level is slightly higher than that of the average serum estrogen levels of sham operated animals, it is still within the physiological estrogen range of female rats during oestrus. The specific estrogen receptor antagonist ICI 182780 had no effect on the serum estrogen levels (as would be expected), but inhibited the rescue of uterus atrophy, showing that the estrogen receptor antagonist specifically antagonised estrogen-receptor dependent estrogen effects.

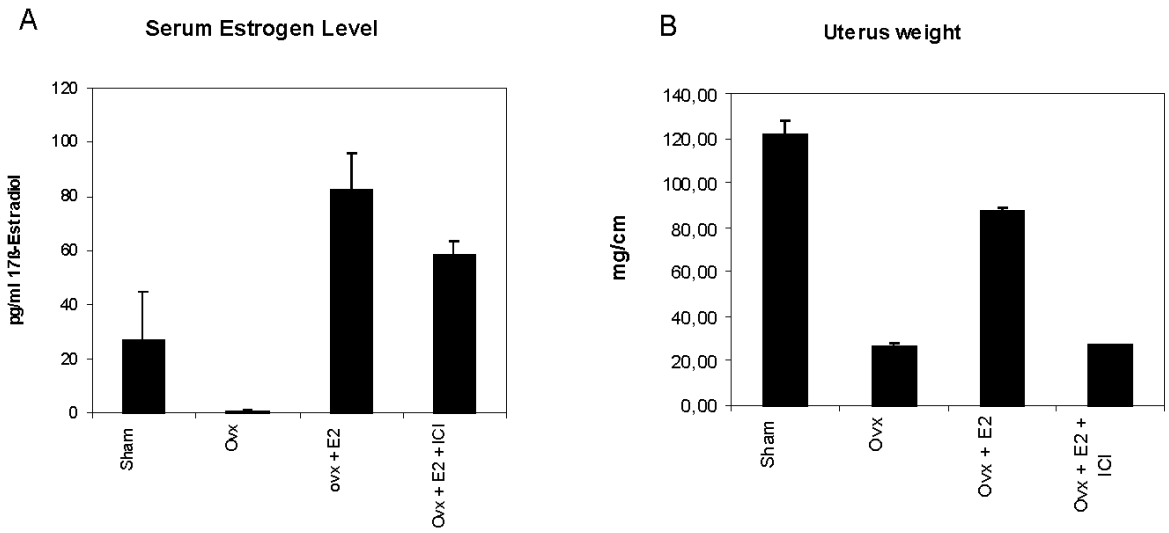


Figure 4: Serum estrogen levels and uterus weight of sham operated and ovarectomised animals to control for efficiency of ovarectomy and estrogen substitution.

A) Ovarectomy (Ovx) resulted in an almost complete depletion of serum estrogen levels. Treatment with 2 µg/kg body weight 17β-estradiol per day (Ovx + E2) over a period of 3 months resulted in restored serum estrogen levels comparable to female animals during oestrus (n=8; $p \leq 0.001$); hence the substitution did not result in supraphysiological serum estrogen levels. ICI 182780 had no significant influence on the serum estrogen levels (Ovx + E2 + ICI). B) Ovarectomy (Ovx) resulted in a reduction of uterus size (n=8; $p \leq 0.001$), which was successfully restored by estrogen-treatment (Ovx + E2; n=8; $p \leq 0.001$). The estrogen receptor antagonist ICI 182780 specifically inhibited the effect of estrogen on uterus size (Ovx + E2 + ICI; n=8; $p \leq 0.001$).

4.1.2 Blood pressure

Since a change in blood pressure is one of the main factors influencing cardiac hypertrophy and therefore indirectly exerts effects on myocardial gene expression, it was important to measure the blood pressure of all the animals before they were sacrificed. Ovarectomy resulted in a reduction of blood pressure from 178 mmHg to 170 mmHg (Figure 5), whereas estrogen substitution resulted in an increase in blood pressure (average 183 mmHg) which was prevented by the estrogen receptor antagonist ICI 182780 (171 mmHg). This showed that the effect of estrogen on the increased blood pressure was specifically mediated in an estrogen receptor-dependent manner.

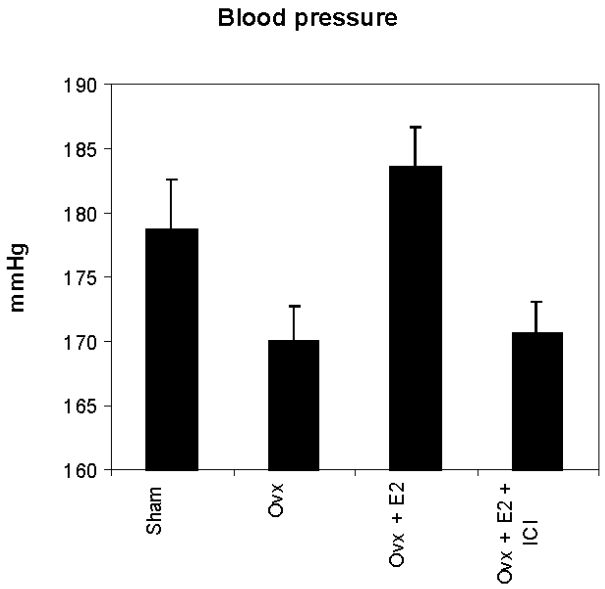


Figure 5: Blood pressure of SHR animals measured after 3 months of estrogen treatment.

Ovarectomy (Ovx) resulted in a reduction of blood pressure compared to sham operated control animals (Sham; n = 8; p = 0.038). Estrogen treatment (Ovx + E2) increased blood pressure (n = 8; p ≤ 0.001), which was inhibited by the estrogen receptor antagonist ICI 182 780 (Ovx + E2 + ICI; n = 8; p ≤ 0.001).

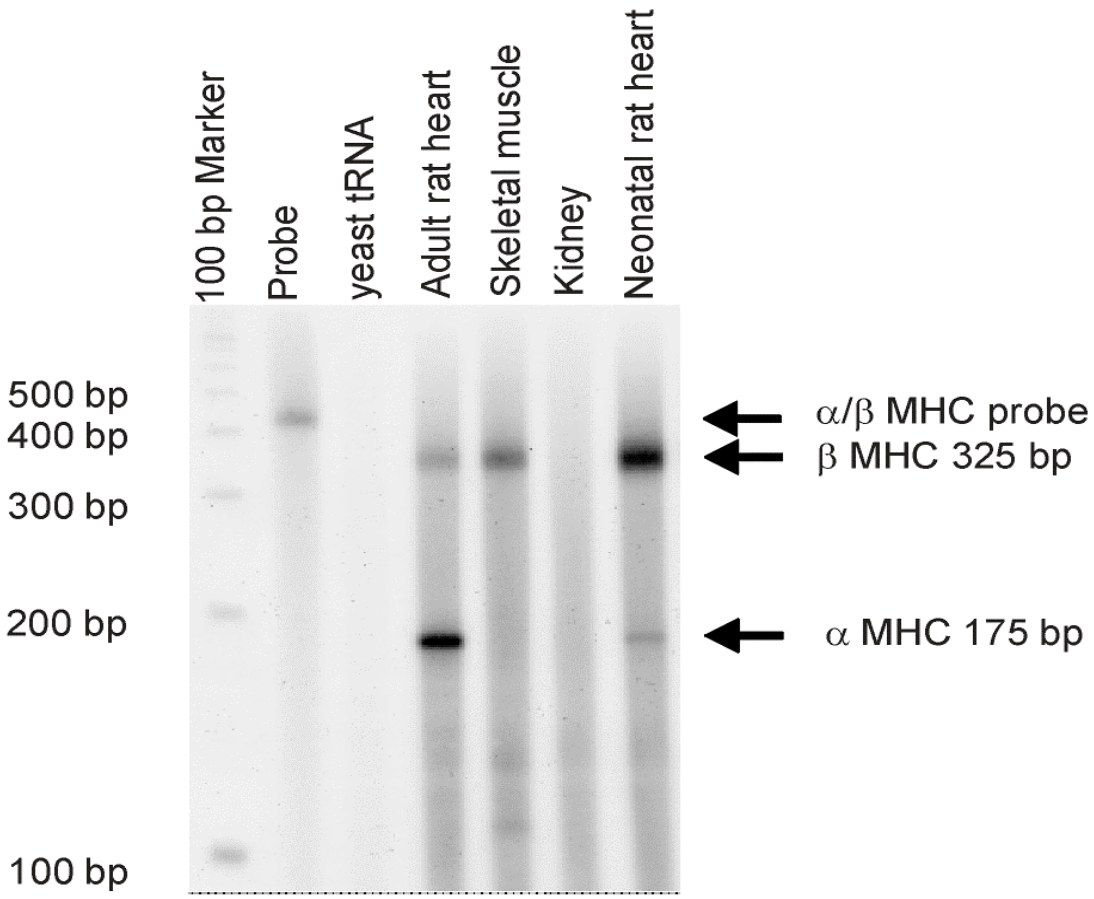
4.1.3 In vivo regulation of contractile gene expression: α-myosin heavy chain expression is regulated by estrogen

4.1.3.1 mRNA expression

The expression of α- and β-MHC mRNA was analysed by RNAse protection assay (RPA), using an *in vitro* transcribed RNA probe that hybridised to both α- and β-MHC mRNAs in one assay (see Methods and Materials section 3.2.3 for details). This technique made it possible to directly compare the amount of α- and β-MHC mRNA in one sample. Included in the assay was an antisense probe to 28S rRNA which was used to standardise the measurements. The specificity of the probe for α- and β-MHC was tested (Figure 6) using mRNAs prepared from normotensive adult rat heart, neonatal rat heart, skeletal muscle and kidney. The control experiment showed that the probe could be successfully used to measure the absolute amounts of α- and β-MHC mRNA levels: in normotensive adult heart more α-MHC than β-MHC was detected, in neonatal rat heart more β-MHC than α-MHC was detected, in skeletal

muscle only β-MHC was detected and in kidney no signal was detected. Phosphoimager analysis of this RPA was performed and the α-/β-MHC ratios calculated, as shown in figure 6. The calculated ratios reflected the observations on the autoradiogram.

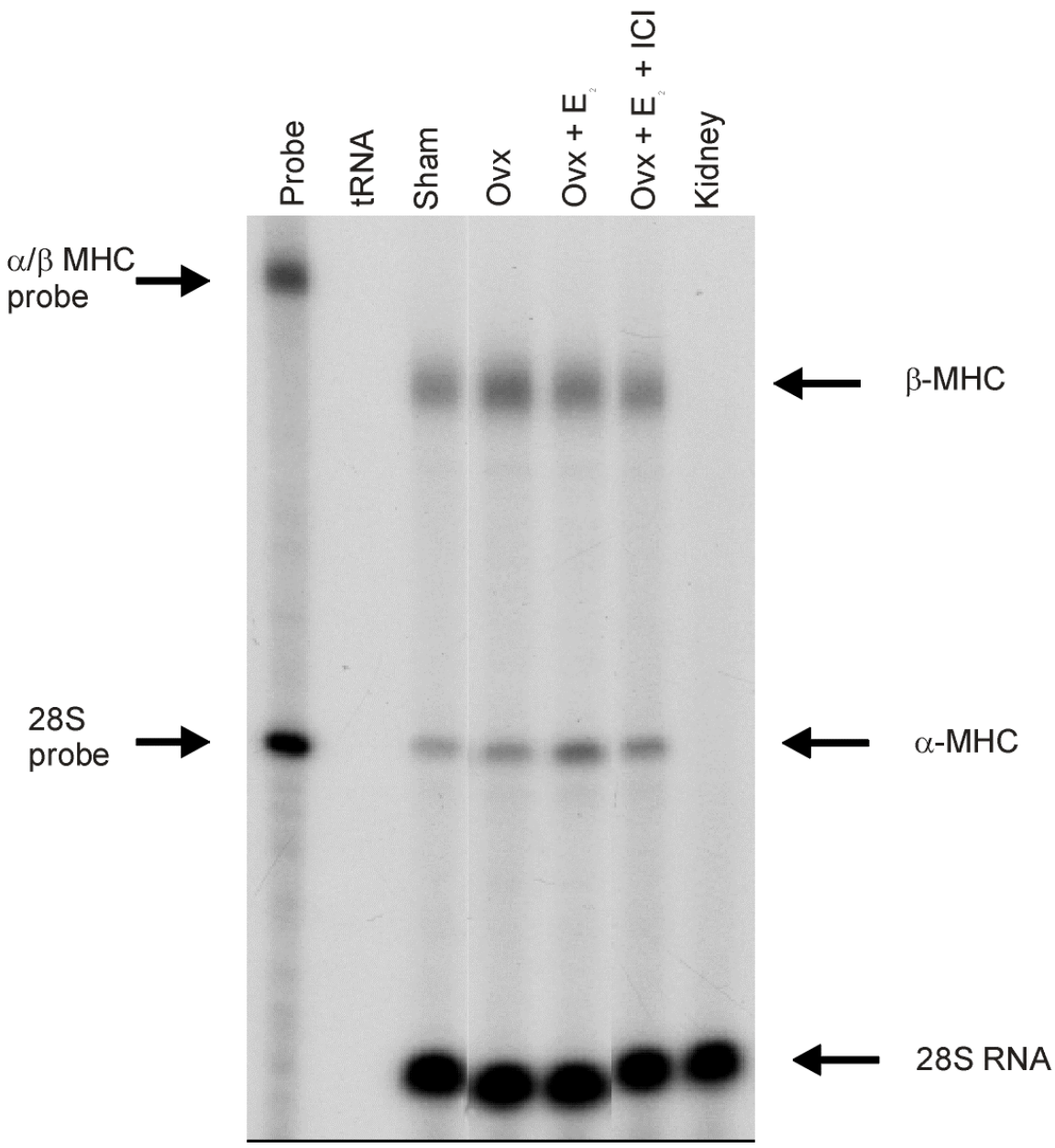
RPA analyses of mRNA prepared from the ventricles of SHR animals included in the study were performed (Figure 7). Phosphoimager analyses of the absolute amounts of α- and β-MHC mRNA levels, showed that ovarectomy resulted in a 32 % decrease in the ratio α- to β-MHC (α-/β-MHC; Figure 8). This decrease in ratio was the result of an upregulation of β-MHC mRNA and a slight downregulation of α-MHC mRNA (Figure 9). Estrogen-treatment resulted in an 83% increase in the ratio α-/β-MHC when compared to ovarectomised animals treated with peanut oil alone, showing that estrogen treatment restored the MHC ratios to an even higher level than sham operated control animals. This shift in the ratio towards α-MHC was the result of an upregulation of α-MHC mRNA by estrogen (+32 % when compared to ovarectomised animals) and a downregulation of β-MHC mRNA (-31% when compared to ovarectomised animals). The shift in the α-/β-MHC ratio upon estrogen-treatment was inhibited by the specific estrogen receptor antagonist ICI 182780, showing that this effect was specifically mediated by the estrogen receptors.



% β-MHC	**28**	**100**	**0**	**70**
% α-MHC	**72**	**0**	**0**	**30**

Figure 6 : RNase protection assay of control RNAs.

The expression of α- and β-MHC mRNA was measured using a single antisense riboprobe that protects β-MHC mRNA over a length of 325 bp as well as α-MHC mRNA over a length of 175 bp. Controls for specificity included yeast t-RNA, normotensive adult rat heart, skeletal muscle, kidney and isolated neonatal cardiomyocytes.



% β-MHC	**65**	**64**	**53**	**69**	**0**
% α-MHC	**35**	**36**	**47**	**31**	**0**

Figure 7: A representative RNAse protection assay of the SHR animals.

The expression of absolute amounts of α- and β-MHC mRNA in the ventricles of SHR animals was measured. An antisense riboprobe directed against 28S rRNA was used to standardise measurements. Yeast t-RNA and mRNA from kidney was used as negative controls. Abbreviations: ovarectomy (ovx), 17-β-estradiol (E_2), ICI 182780 (ICI). Densitometric analysis of % α-MHC and %β-MHC is shown in the table.

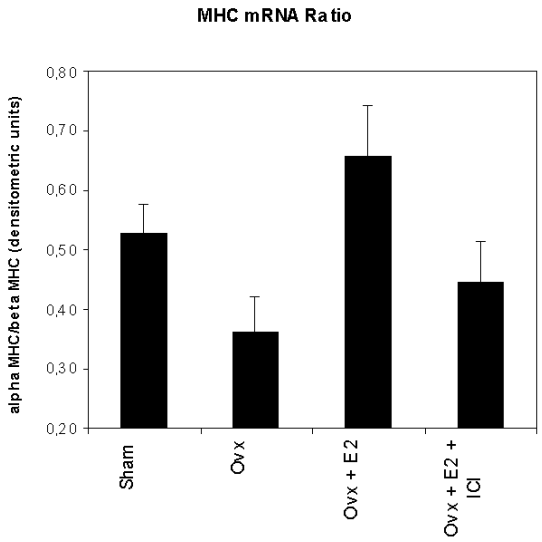


Figure 8: Ratios of α- and β-MHC mRNAs expressed in hearts of sham operated and ovarectomised animals.

The absolute amount of α- and β-MHC mRNA was densitometrically measured after RPA using 28S rRNA as an internal standard. The ratios of absolute α-MHC to β-MHC were calculated and are shown here. Ovarectomy (Ovx) resulted in a reduction of MHC ratio (n = 8, n.s.), whereas estrogen treatment increased the ratio towards more α-MHC (Ovx + E2; n = 8; $p \leq 0.05$). This effect was inhibited by the specific estrogen receptor antagonist ICI 182780 (Ovx + E2 + ICI; n = 8; $p \leq 0.05$).

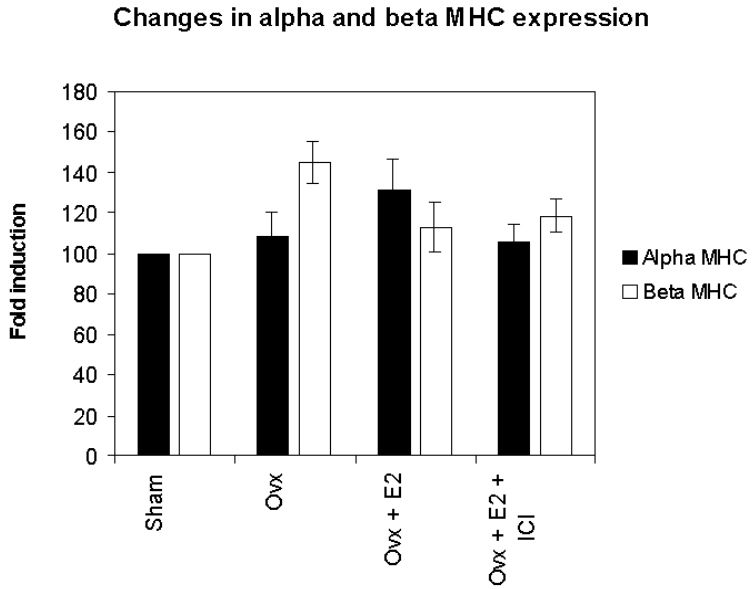


Figure 9: Changes in expression of α- and β-MHC in sham and ovarectomised animals.

Estrogen-treatment of ovarectomised animals resulted in an upregulation of α-MHC (n = 8; $p \leq 0.05$) and a subsequent downregulation of β-MHC (Ovx + E2; n = 8; $p \leq 0.05$) . These changes in α- and β-MHC expression were prevented by the specific estrogen receptor antagonist ICI 82780 (Ovx + E2 + ICI; n = 8; $p \leq 0.05$).

4.1.3.2 Protein expression

Denaturing gel electrophoresis of unfractionated protein samples prepared from ventricles of SHR animals allowed the separation of α- and β-MHC isoforms (Figure 10). Densitometric analyses of the separated MHC isoforms supported the mRNA data: ovarectomy resulted in a reduction of the α- /β-MHC ratio when compared to sham operated controls, and estrogen substitution of ovarectomised animals resulted in an increase in α-/β-MHC ratio (Figure 11). This increase in α- /β - MHC ratio was mainly the result of an upregulation of the amount of α-MHC protein. The estrogen receptor antagonist ICI 182780 prevented this estrogen induced increase in α-/β-MHC ratio in ovarectomised animals, again providing evidence for a specific estrogen receptor dependent estrogen action in the myocardium.

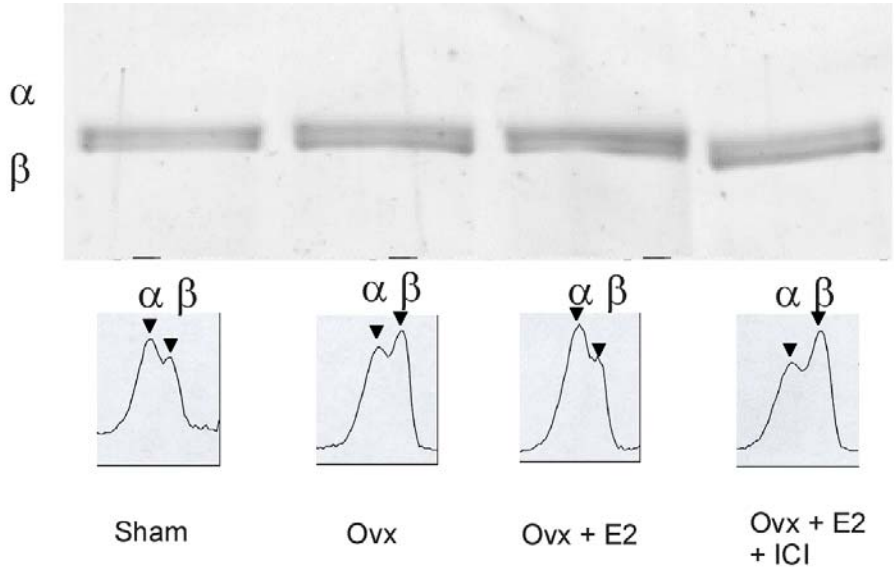


Figure 10: A representative silver stained SDS gel after separation of α- and β-MHC isoforms in SHR animals.

The upper band represents α-MHC and the lower band β-MHC, as verified by Western blotting. Densitometric analyses of α- and β-MHC isoforms are shown in the lower panel. From the densitometric analyses it is clear that ovarectomy (Ovx) resulted in a decrease in α-MHC protein and an increase in β-MHC protein expression. Treatment of ovarectomised animals with 2 µg/kg body weight 17β-estradiol (Ovx + E_2) per day resulted in an increase in α-MHC protein and a decrease in β-MHC, which was inhibited by the specific estrogen receptor antagonist ICI 182780 (Ovx + E_2 +ICI).

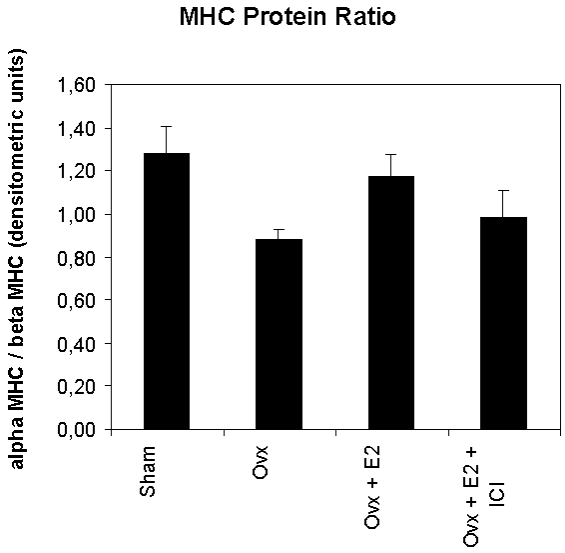


Figure 11: Ratios of α- and β-MHC protein expressed in hearts of sham operated and ovarectomised animals.

α- and β-MHC protein was separated in SDS containing non-reducing gels and the ratios of α-/β-MHC expression was densitometrically measured. Ovarectomy (Ovx) resulted in a shift in ratio towards less α-MHC, whereas estrogen substitution (Ovx + E2) prevented this shift in MHC ratio. ICI antagonised the estrogen effect (Ovx + E2 + ICI).

Western blotting experiments confirmed and supported the results of the gel electrophoresis (Figure 12). However, due to different antibodies used for the detection of α- and β-MHC protein, it is not possible to calculate the ratios using this technique.

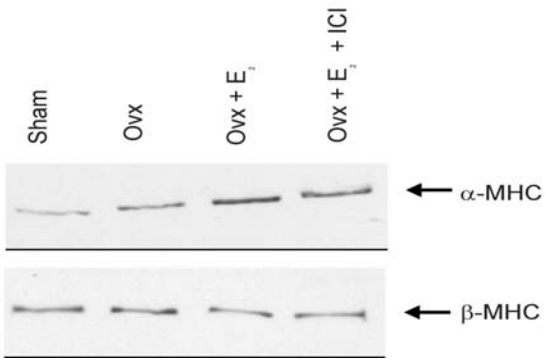


Figure 12: Western blot of α- and β-MHC protein expression.

The bands shown in figure 10 was verified as α-and β-MHC isoforms by western blotting using specific antibodies directed against the two isoforms. The results of the SDS gel electrophoresis was confirmed by Western blot.

In summary it was shown in this animal study, that estrogen has a direct *in vivo* effect on the myocardium by regulating the expression of one of the major contractile proteins and its mRNA, the myocardial specific α-MHC. This effect of estrogen is specifically mediated by estrogen receptors since it can be reversed by the estrogen receptor antagonist ICI 182780. It was therefore shown that estrogen receptors are functional *in vivo* in the myocardium and play an important role in mediating specific myocardial estrogen effects.

4.2 *In vitro* regulation of α - myosin heavy chain expression by estrogen in isolated cardiomyocytes

The regulation of α-MHC expression by estrogen was analysed in cell culture using an established model of isolated primary neonatal rat cardiomyocytes. This cellular system allowed investigations into the mechanism of estrogen action in the absence of other external factors such as high blood pressure and cardiac hypertrophy or other cell types such as fibroblasts and vascular cells.

4.2.1 In vitro regulation of α - MHC mRNA expression

Isolated cardiomyocytes were cultured under steroid hormone-free conditions before experiments were initiated. Treatment of primary neonatal cardiomyocytes with 10 nM 17β-estradiol for 72 hours resulted in a 48% increase in the ratio α-/β-MHC ($n = 7$, $p<0.05$) which was inhibited by the specific estrogen receptor antagonist ICI 182780 (Figure 13). This increase in ratio was the result of a 28% upregulation of α-MHC mRNA ($n=7$; $p<0.05$) and a subsequent 13% downregulation of β-MHC mRNA (Figure 14; $n=7$; $p<0.02$), as measured by RPA followed by phosphoimager analysis. The effect of estrogen on α- and β-MHC expression was specifically inhibited by ICI 182780, showing an estrogen receptor dependent regulation in isolated cardiomyocytes. These data also confirmed that the upregulation of α–MHC observed in the animal study was specifically due to a direct estrogen action on the myocardium.

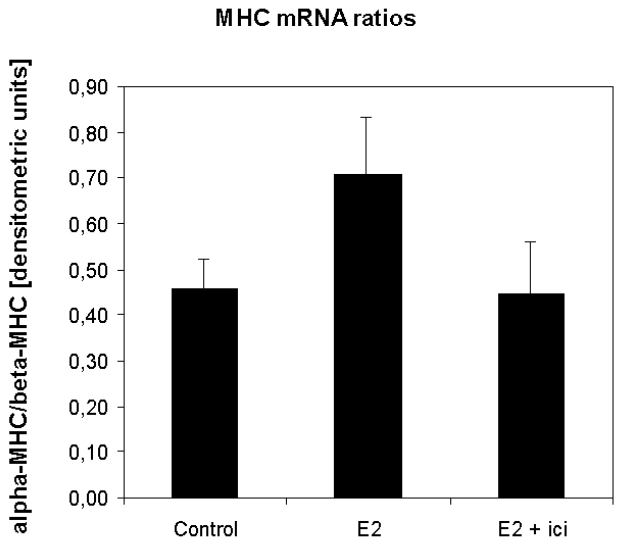


Figure 13: Ratios of α- and β-MHC in isolated primary neonatal cardiomyocytes.

Neonatal cardiomyocytes were treated with 10 nM 17 β-estradiol (E2) lone or in the presence of 1 µM ICI 182780 (E2 + ICI) for 72 hours. Absolute amounts of α- and β-MHC mRNAs were measured by RPA and phosphoimager analyses. The ratios of α-MHC to β-MHC are shown here as densitometric units.

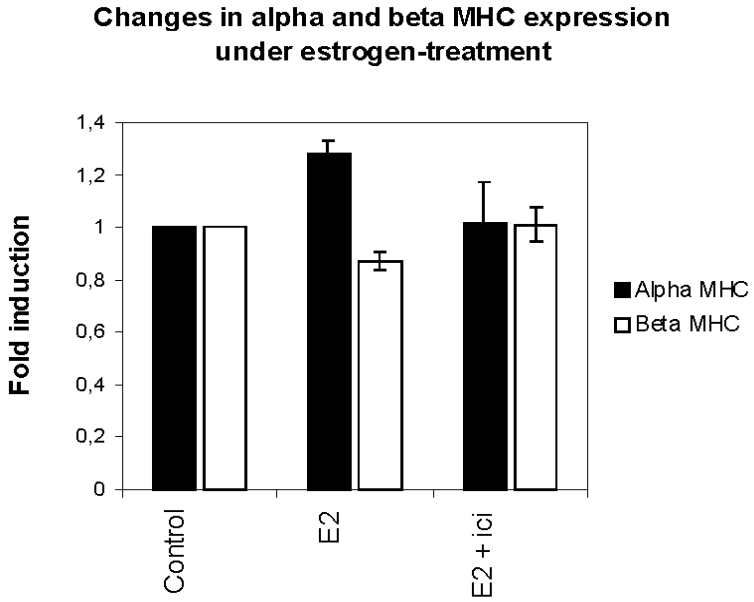


Figure 14: Changes in α-MHC and β-MHC mRNA expression under estrogen-treatment.

Treatment of isolated neonatal cardiomyocytes with 10 nM E_2 for 72 hours resulted in an upregulation of α-MHC expression (n = 7; $p \leq 0.05$) and a subsequent downregulation of β-MHC expression (n = 7; $p \leq 0.02$). These changes in α- and β-MHC expression were inhibited by the specific estrogen receptor antagonist ICI 182780 (1 µM) Results are presented as fold induction over control (untreated cardiomyocytes).

4.2.2 In vitro regulation of α - MHC protein expression

Densitometric analyses of α- and β-MHC protein isolated from primary neonatal cardiomyocytes treated with 10 nM E_2 for 72 hours showed that the estrogen-treatment resulted in a shift in MHC ratio towards more α-MHC (Figure 15 and 16). This shift was inhibited by the estrogen receptor antagonist ICI 182780 (1 µM). The changes in α-/β-MHC ratios were due to an upregulation of α-MHC protein expression and a subsequent downregulation of β-MHC protein expression. These results in isolated cardiomyocytes are in cohort with the mRNA expression patterns as well as with the observations in the long term animal study.

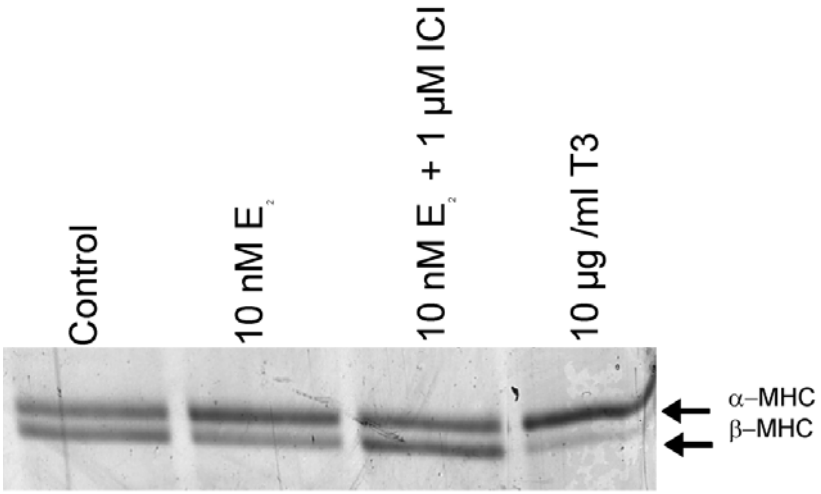


Figure 15: A representative silver stained SDS gel after separation of α- and β-MHC proteins in isolated neonatal cardiomyocytes.

Isolated neonatal cardiomyocytes were treated with 10 nM E_2 alone or in combination with 1 µM estrogen receptor inhibitor ICI 182780. 10 µg/ml T3 served as positive control for the upregulation of α-MHC protein expression. Estrogen-treatment resulted in a specific estrogen receptor-dependent upregulation of α-MHC protein expression, shifting the α-/β-MHC ratio towards more α-MHC.

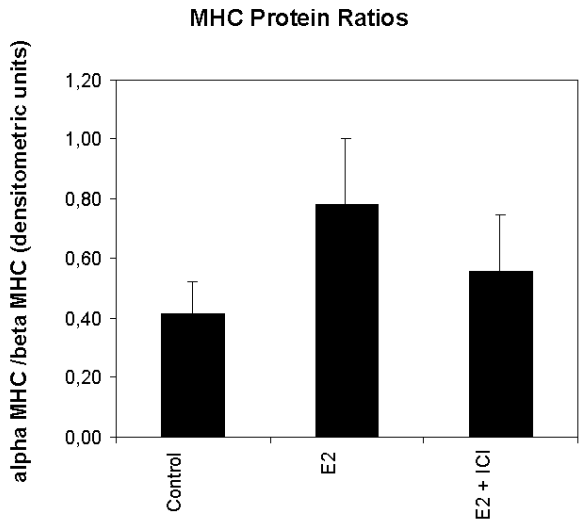


Figure 16: Ratios of α- and β-MHC protein expression in isolated primary neonatal cardiomyocytes.

Neonatal cardiomyocytes were treated with 10 nM E_2 for 72 hours alone or in the presence of 1 µM ICI 182780. Proteins were separated in SDS gels, silver stained and the ratios of α-/β-MHC expression measured. Estrogen-treatment resulted in a shift in ratio towards more α-MHC, which was inhibited by ICI 182780 treatment. Shown here are α- /β-MHC ratios as densitometric units.

4.3 Investigations into the mechanism of α - myosin heavy chain regulation by estrogen in the myocardium

4.3.1 In vitro regulation of the α - MHC promoter

To investigate whether 17β-estradiol directly induce the α-MHC promoter, the 5.4 kb mouse α-MHC promoter cloned in front of a luciferase reporter gene (α-MHC-luc), was transfected into primary neonatal cardiomyocytes. After transfection, cells were either treated with medium alone (control) or with 100 nM 17β-estradiol alone or in combination with 1 µM ICI 182780 (Figure 17) for 48 hours. Thyroid hormone (10 mg/ml T3, 3,3',5-triiodo-$_L$-thyonine), which is one of the strongest inducers of α-MHC, served as positive control. T3–treatment resulted in a 114% upregulation of α-MHC promoter activity. Estrogen–treatment resulted in an 88% upregulation of α-MHC promoter activity (n=6, p=0.05) which was completely inhibited by ICI 182780, showing an estrogen receptor – dependent mechanism of α - MHC

upregulation by estrogen. ICI 182780 treatment alone had no significant effect on α - MHC promoter activity.

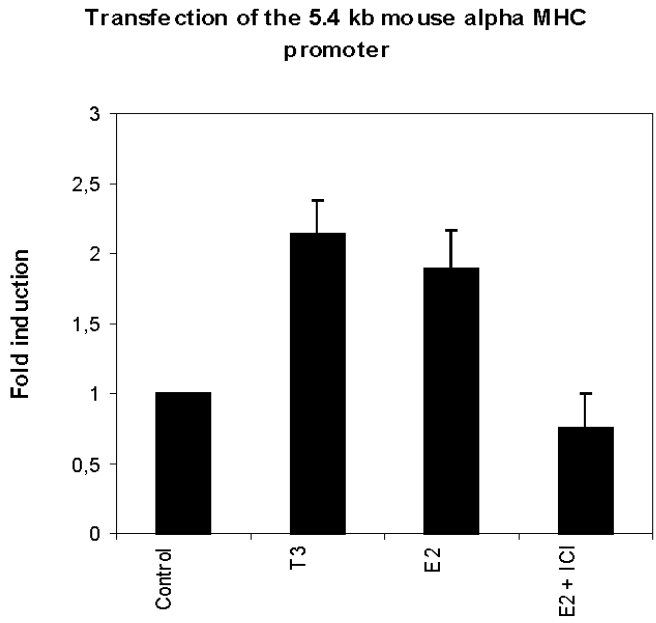


Figure 17: Transfection of the mouse α-MHC promoter in primary neonatal rat cardiomyocytes.

Shown here is the fold induction over control of transfected cardiomyocytes after luciferase measurements. 10 µg/ml T3 resulted in a 2.3 fold induction of α-MHC promoter. Estrogen treatment induced the promoter by 85% (E2; n = 6; p = 0.05) which was completely inhibited by 1 µM ICI 182780 (E2 + ICI).

4.3.2 Co -transfection experiments with Egr-1

One study has indicated Egr-1, a zinc finger transcription factor, as important transcription factor for the induction of α-MHC. Potential binding sites for Egr-1 in the α-MHC promoter have also been identified. Our group has previously shown that Egr-1 is strongly and rapidly induced by estrogen and investigations into the mechanism of Egr-1 regulation by estrogen is described in another section of this thesis. The question was investigated whether the induction of α-MHC by estrogen is mediated via an induction of Egr-1 protein and subsequent binding of Egr-1 to the α-MHC promoter, leading to enhanced α-MHC expression. To answer this question, Egr-1 cDNA under the control of the CMV promoter was co-expressed with the α-MHC promoter construct in primary neonatal cardiomyocytes. The lacZ cDNA under the control of the CMV promoter co-expressed with the α-MHC

promoter served as negative control. Co-expression of Egr-1 cDNA together with the α–MHC promoter resulted in an upregulation of α-MHC promoter activity of 52% (n = 4; Figure 18), which is comparable to the upregulation of promoter activity by estrogen alone (Figure 17). This result, therefore, serve as the first indication that Egr-1 might be involved in one of potentially many mechanisms by which α-MHC is regulated by estrogen.

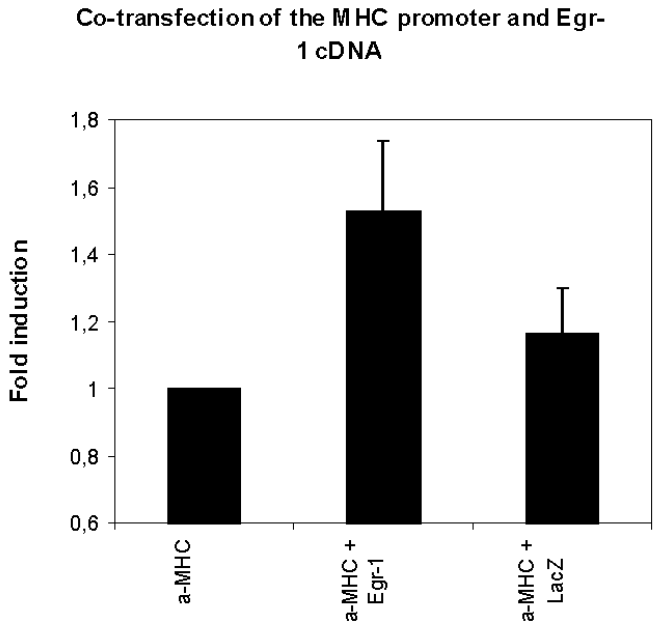


Figure 18: Co-transfection of Egr-1 cDNA and the mouse α-MHC promoter in primary neonatal cardiomyocytes.

Egr-1 cDNA under the control of the CMV promoter was co-transfected with the mouse α-MHC promoter (a-MHC + Egr-1) and is shown as fold induction over control (a-MHC). The LacZ cDNA under the control of the same promoter was used as negative control (a-MHC + LacZ). Egr-1 co-expression resulted in an increase of α-MHC promoter activity comparable to the induction of the promoter by estrogen treatment. LacZ cDNA had no significant effect on the α-MHC promoter activity.

In summary it was shown, that the *in vivo* regulation of α-MHC expression in the intact heart could be confirmed in isolated cardiomyocytes treated with physiological concentrations of 17β-estradiol, providing further evidence for a direct estrogen receptor-mediated estrogen action on cardiomyocytes. It was furthermore shown that estrogen induced the α-MHC promoter and first experiments to characterise the mechanism of this estrogen action, showed that an Egr-1 mediated pathway might be involved.

4.4 Mechanisms of rapid estrogen action in cardiomyocytes

Various studies on the effects of estrogen in different tissues have shown that rapid, so-called “non-genomic” effects of estrogen play a major role in the regulation of gene expression as well as protein activity. In a previous study in our laboratory it was shown that an immediate early gene, the early growth response gene –1 (Egr-1) is highly and rapidly induced by estrogen in cardiomyocytes in an estrogen receptor-dependent manner (Shamim thesis). A detailed promoter analysis study of the Egr-1 promoter has shown that Egr-1 is regulated by estrogen not via the ERE half-sites in the upstream promoter region, but via a promoter region containing mainly serum response elements (SREs) (see Figure 19).

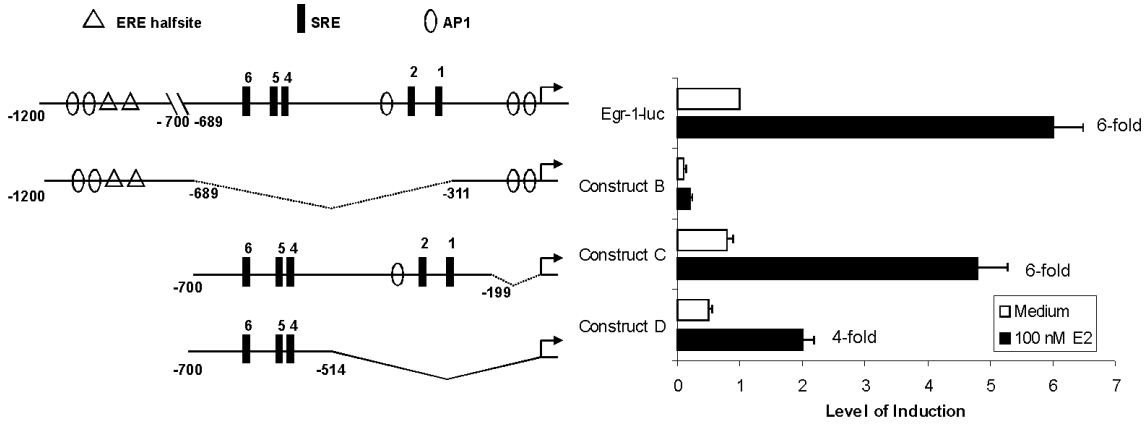


Figure 19: Egr-1 promoter analysis to identify the sites responsible for the estrogen-inducibility.

A detailed analysis of the Egr-1 promoter showed that the estrogen-inducibility was not mediated via the ERE, but through a promoter region containing mainly a cluster of SREs. Modified from PhD Thesis, Shamim, University of Wuerzburg, 1999.

The question to the mechanism of this induction by estrogen arose, since a direct binding of estrogen receptors to the ERE half-sites do not seem to play a significant role in the induction by estrogen. In this study, the analysis of the Egr-1 induction by estrogen was continued to understand the mechanism involved in the induction by estrogen. The following questions were addressed: 1) What signalling pathways are involved in the rapid induction of Egr-1 by estrogen? 2) Can an artificial promoter containing only SREs be induced by

estrogen in an estrogen receptor-dependent manner? 3) Does estrogen-treatment recruit transcription factors to the SREs in the Egr-1 promoter?

4.4.1 Rapid activation of signal transduction pathways

4.4.1.1 ERK1/2

Isolated primary neonatal cardiomyocytes were treated with 10 nM of 17β-estradiol for 5, 15, 30 and 60 minutes and the phosphorylation of ERK1 and ERK2 was examined using a phospho-specific antibody (Figure 20). In cardiomyocytes a difference in the phosphorylation patterns of ERK1 and ERK2 was detected after estrogen-treatment. Both ERK1 and ERK2 were rapidly and transiently phosphorylated by estrogen-treatment, but a stronger phosphorylation of ERK2 than ERK1 was detected. ERK1-phosphorylation declined already after 15 minutes of treatment and after 30 minutes no phosphorylated ERK1 was detected. This resembles the phosphorylation pattern observed after phorbol-myristate-acetate (PMA) treatment. However, ERK2 was strongly phosphorylated by estrogen-, FCS- and PMA-treatment which was still detectable after 60 minutes of treatment. These results showed that estrogen rapidly and transiently activates ERK1 and ERK2 and that the phosphorylation patterns were different for the two kinases.

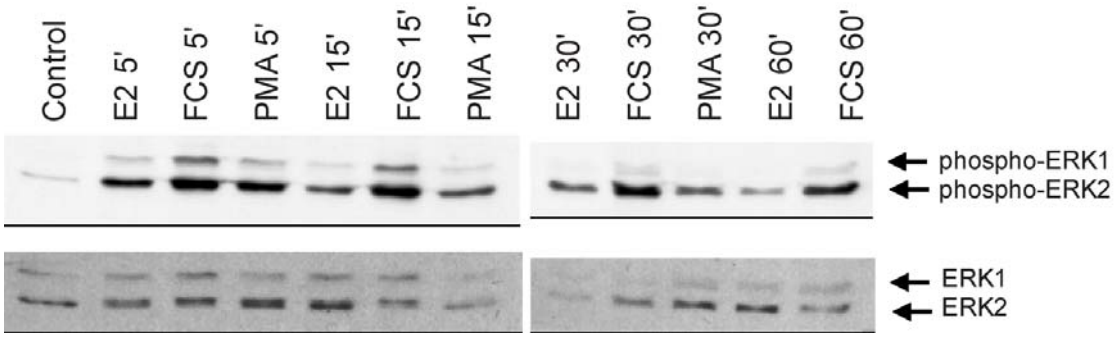


Figure 20*: *Western blot analysis of the phosphorylation of ERK1/2.

Estrogen-treatment resulted in the rapid phosphorylation of ERK1/2 after 5 minutes. Treatment of primary neonatal cardiomyocytes with 20% FCS and 200 ng/ml PMA served as positive controls. Expression of ERK1 and 2 protein was largely unaltered by estrogen-treatment.

4.4.1.2 p38 Mitogen Activated Protein Kinase

The effects of estrogen treatment on the phosphorylation of the p38 mitogen activated protein kinase (MAPK) was investigated in parallel to ERK1/2. Estrogen-treatment resulted in the rapid and transient phosphorylation of the p38 MAPK. After 15 minutes of estrogen treatment almost no phosphorylated p38 could be detected. This phosphorylation resembles that of ERK1.

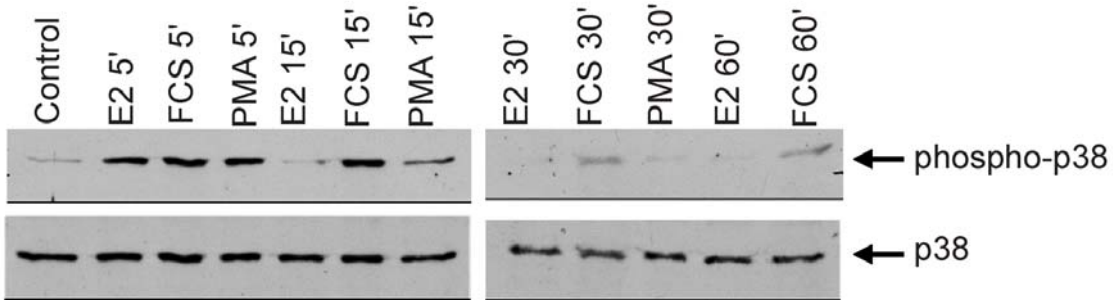


Figure 21: Western blot analysis of the phosphorylation of p38.

Estrogen-treatment resulted in the rapid phosphorylation of the p38 MAPK after 5 minutes. No phosphorylated form of p38 MAPK was detected after 30 minutes of estrogen-treatment. Treatment of primary neonatal cardiomyocytes with 20% FCS and 200 ng/ml PMA served as positive controls. Expression of p38 MAPK protein was unaltered by estrogen-treatment.

4.4.1.3 Rapid induction of the early growth response gene via ERK1/2

Since the mechanism of the rapid induction of Egr-1 by estrogen (maximum induction after 15 minutes on mRNA level and after 90 minutes on protein level) was not clear, it was investigated whether the induction of Egr-1 by estrogen is mediated via the signal transduction pathways activated by estrogen in an estrogen receptor-dependent manner as shown in sections 4.4.1.1 and 4.4.1.2. Primary neonatal cardiomyocytes were treated with 10 nM of 17β-estradiol alone or in combination with 1 µM ICI 182780, a specific estrogen receptor antagonist, or PD 98059, a specific inhibitor of ERK1/2, or SB 202190, a specific inhibitor of the p38 MAPK. Estrogen-treatment resulted in a strong upregulation of Egr-1 protein after 90 minutes of treatment which was inhibited by both ICI 182780 as well as PD

98059, but not by SB 202190 (Figure 22). This result showed that Egr-1 is induced by estrogen in an estrogen receptor dependent manner which is mediated via ERK1/2 but not via the p38 MAPK.

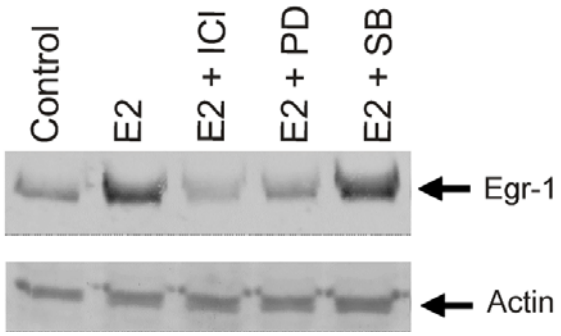


Figure 22: Egr-1 is rapidly induced by 17β-estradiol in an estrogen receptor-dependent manner in primary neonatal cardiomyocytes.

The induction of Egr-1 protein was detected by Western blot analysis of total protein isolated from primary neonatal cardiomyocytes after 90 minutes of estrogen treatment with a specific antibody directed against Egr-1. Actin served as loading control. The induction of Egr-1 protein by E_2 was completely inhibited by ICI 182780 (1 µM) and by the MEK inhibitor PD 98059 (10 µM), but not by the p38 MAPK inhibitor SB 202190 (10 µM).

4.4.1.4 Estrogen induces target gene expression via serum response elements

A detailed promoter analysis of the Egr-1 promoter clearly showed that a region of the Egr-1 promoter containing mainly SREs and no EREs or Ap-1 sites, is necessary for the induction by estrogen (Shamim, PhD Thesis). To test whether SREs alone are sufficient for the induction of a promoter by estrogen, an artificial promoter containing 5 SREs linked to luciferase (pSRE-luc) was transfected into primary cardiomyocytes. Estrogen-treatment resulted in a 3.3-fold induction of this construct (Figure 23; n=5, $p \leq 0.05$) which was specifically inhibited by the estrogen receptor antagonist ICI 182780. ICI –treatment alone had no effect on the pSRE-luc. This result therefore provided evidence that SREs alone can confer an estrogen response to a heterologous promoter.

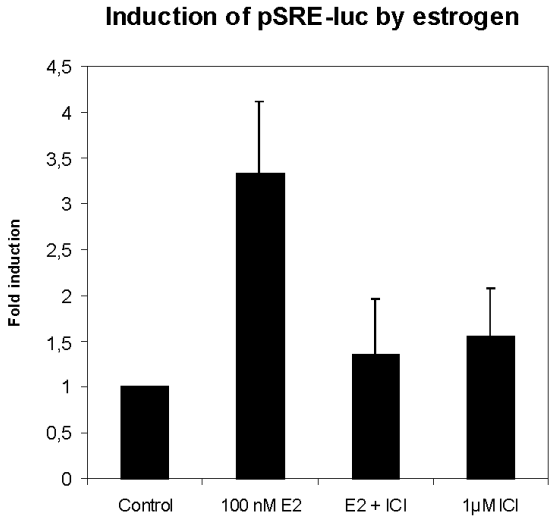


***Figure 23*: *17β-estradiol induces an artificial promoter containing 5 X SREs*.**

Estrogen-treatment (E2) resulted in a 2.3-fold induction of pSRE-luc (n=5, p≤ 0.05), which was completely inhibited by the specific estrogen receptor antagonist ICI 182780 (E2 + ICI). ICI alone had no effect on the expression if the construct.

4.4.1.5 Estrogen receptor-α as well as estrogen receptor-β dependent induction of target genes via SREs

Overexpression of ERα as well ERβ resulted in a 9- and 6.5-fold induction of the artificial SRE-containing promoter construct (Figure 24). This induction by estrogen was inhibited by the specific estrogen receptor antagonist ICI 182780, showing that the induction was specifically mediated via the overexpressed estrogen receptors. No significant difference between the induction via ERα and ERβ was observed.

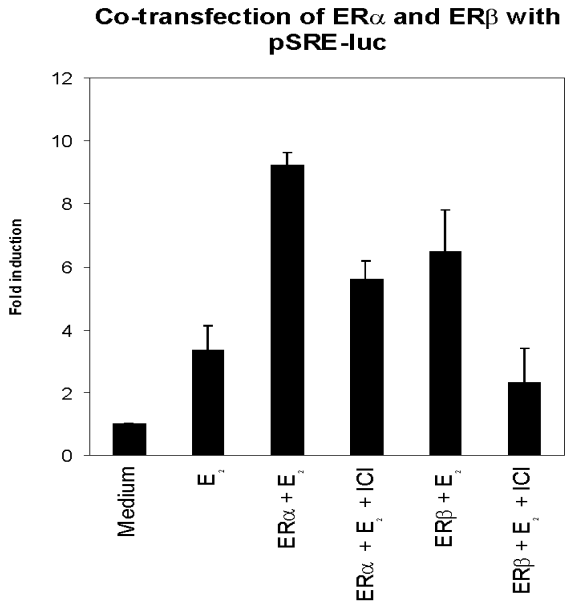


Figure 24: ERα as well as ERβ overexpression resulted in the induction of a heterologous SRE-containing promoter construct.

Co-transfection of ERα cDNA with pSRE-luc resulted in a 9-fold induction by E_2 ($n = 2$; $p \leq 0.05$) which was inhibited by ICI 182780. Overexpression of ERβ with pSRE-luc resulted in a 6.5-fold induction of this construct by E_2 ($n = 2$; $p \leq 0.05$), which was inhibited by ICI 182780.

4.4.1.6 Estrogen recruits the binding of serum response factor to the serum response elements in the Egr-1 promoter

Electrophoretic mobility shift assays (EMSAs) were performed utilising nuclear extracts prepared from isolated neonatal cardiomyocytes treated with 10 nM E_2 for 15 minutes and untreated controls to investigate whether estrogen-treatment induced the binding of transcription factors to the SREs in the Egr-1 promoter. Oligonucleotide probes matching the 3 upstream SREs in the Egr-1 promoter were used in the EMSAs. The formation of specific protein-DNA complexes was induced by estrogen (Figure 25) indicating that hormone treatment altered the transcription factor recruitment in an estrogen-dependent manner. Supershift assays with polyclonal antibodies directed against the serum response factors (SRF) identified a protein with the antigenic characteristics of SRF as one of the proteins in the protein-DNA complex.

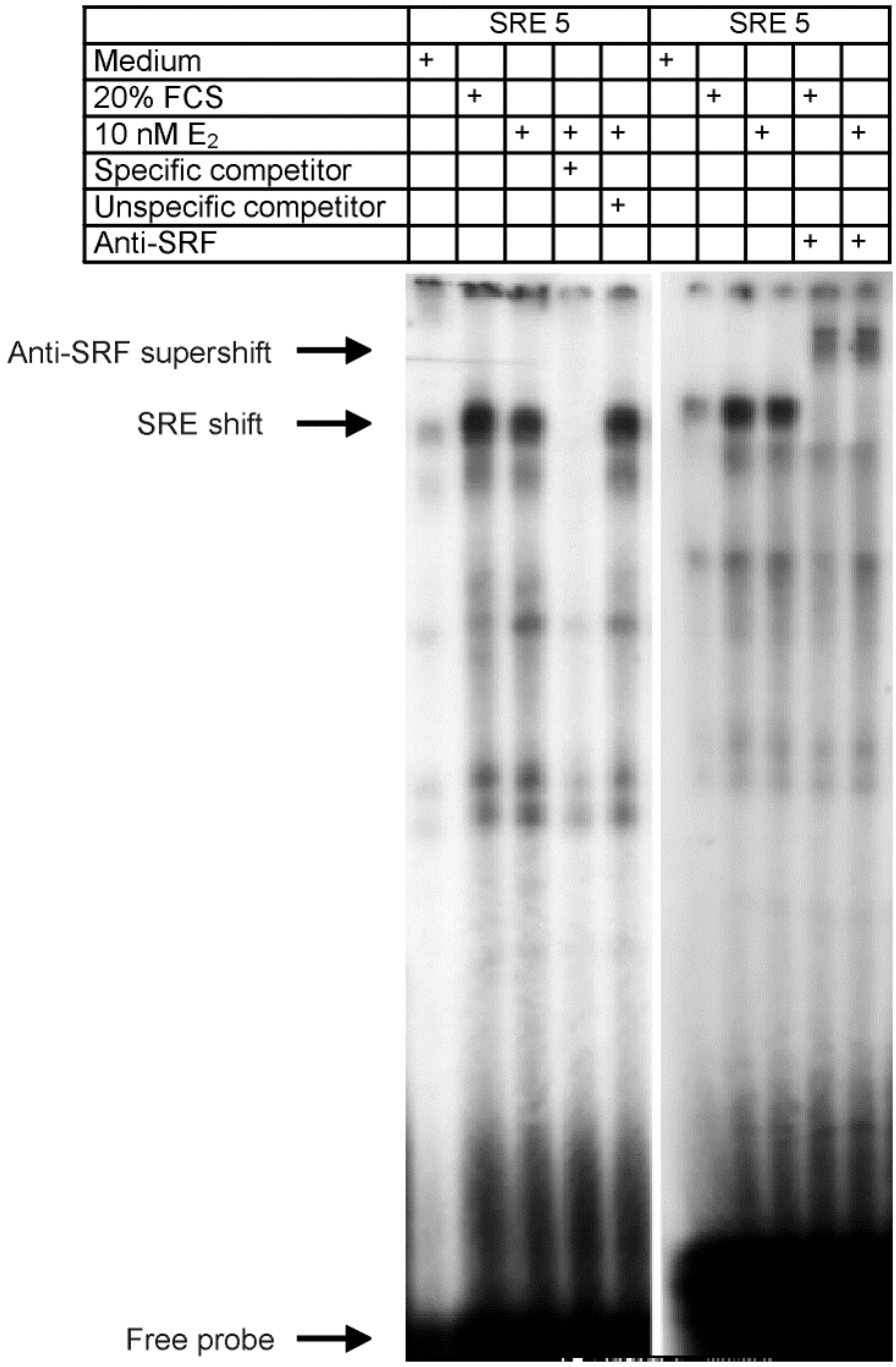


Figure 25: 17β-Estradiol-treatment recruited transcription factors to the SREs in the Egr-1 promoter.

Nuclear extracts of cardiomyocytes treated with 10 nM E_2 resulted in the binding of protein complexes to the SREs. Specific protein-DNA complexes were formed which were competed away with an excess of unlabeled specific oligonucleotide probes, but not by an unspecific oligonucleotide probe. An anti-SRF antibody shifted the SRE complex (Anti-SRF supershift), identifying SRF, or an antigenically related protein, in the complex.

In summary it was shown that estrogen-treatment resulted in the rapid phosphorylation of ERK1/2 and p38 MAPK, showing that estrogen can exert rapid myocardial effects. It was furthermore shown that the rapid induction of an estrogen target gene, Egr-1, by estrogen is mediated via ERK1/2 in an estrogen receptor-dependent manner, but not via the p38 MAPK. Investigations into the mechanism of this activation showed that the estrogen receptor-dependent induction of Egr-1 is mediated via the SREs in the Egr-1 promoter. It was shown that SREs alone are responsive to estrogen as shown by the induction of a heterologous promoter containing only 5 SREs by estrogen. This induction was mediated by both ERα as well as ERβ as shown in co-transfection experiments. Furthermore, it was shown that transcription factor complexes containing SRF are recruited to the SREs upon estrogen-treatment. This study, therefore clarified the mechanisms involved in the rapid induction of Egr-1 by estrogen in cardiomyocytes.

4.5 cDNA Microarray analyses

cDNA microarray analyses were performed using pooled total RNA prepared from the left ventricles of ovarectomised SHR animals compared to RNA prepared from left ventricles of ovarectomised SHR animals that were treated with 17β-estradiol for 3 months (see Methods). Analyses of the filters were performed in duplicate and the genes shown to be regulated in both experiments are summarised in table 1. This is the first indication of potentially novel target genes that might be regulated by estrogen *in vivo*. However, due to the time limits of the study, confirmation of the regulation was not performed on Northern or Western blots and this analysis should be seen as an initiation of the search for novel estrogen targets in the myocardium which will be continued.

The cDNA array filters used for the analyses allowed for the simultaneous analysis of 1250 known genes from the rat. Of the 1250 genes, 24 were found to be changed when analysed with the AtlasImage software (Clontech, Germany). The global background was calculated and the intensities of the single spots above background were measured. The intensities of

the cDNA spots that hybridised to RNA from the ovx + E_2 animals were divided by the intensities of cDNA spots that hybridised to RNA from the ovx animals to calculate the fold induction.

Table 1: Genes modified by estrogen *in vivo* in SHR animals

Nr.	Filter Grid #	Gene	GenBank Acc. #	Fold regulation
Transcription factors and associated genes:				
1	A05i	I-kB (I-kappa B) alpha chain	X63594	10.1
2	A06g	DNA-binding protein inhibitor ID1	D10862	1.7
3	A10j	c-jun proto-oncogene	X17163	1.6
4	A11f	nucleoside diphosphate kinase B; c-myc-related transcription factor	M91597	1.7
Ion channel genes:				
5	B03i	sodium channel SHRSPHD, gamma subunit, epithelial	X77933	0.4
6	B05c	mink potassium channel; KCNE1	M22412	11.1
7	B08b	adenine nucleotide translocator 2 (ANT2)	D12771	Induced*
8	B10b	sodium/potassium-transporting ATPase beta 1 subunit (ATP1B1)	J02701	2.4
Energy metabolism genes:				
9	C03b	medium chain acyl-CoA dehydrogenase precursor	J02791	3.3
10	C03l	cytochrome c oxidase, subunit VIIIh	X64827	0.8
11	C04a	mitochondrial ATP synthase D subunit; ATP5H	D10021	89.0
12	C04b	mitochondrial ATP synthase beta subunit precursor (ATP5B)	M19044	2.6

13	C07f	cytochrome P450 4A3 (CYP4A3)	M33936	Induced*
14	C09k	long chain-specific acyl-CoA dehydrogenase precursor	J05029	1.9
Ribosomal genes:				
15	C11g	ribosomal protein L11	X62146	4.1
16	C11h	ribosomal protein L13	X78327	4.9
Other genes:				
17	C11b	calcium binding protein 2 (CABP2)	M86870	Induced*
18	C12d	Elongation factor 2 (EF2)	K03502	3.9
19	C12g	Eukaryotic translation initiation factor 2 alpha subunit (EIF-2-alpha)	J02646	8.4
20	D14h	beta-nerve growth factor precursor (beta-NGF)	D28498	0.4
21	E09e	cell adhesion kinase beta (CAK beta)	D45854	Induced*
22	F08k	Metalloendopeptidase meprin beta subunit	M88601	1.7
23	A01g	Annexin V (ANX5)	M21730	3.5
24	A03g	CD4 Homologue	M15768	4.0
* - these genes were not detectable in animals untreated with estradiol, i.e. they were switched on by estradiol-treatment				

5 Discussion

The present study focussed on the effects of estrogen on myocardial gene expression *in vivo* as well as *in vitro*. In the first part of the study it was shown, that estrogen-treatment resulted in the upregulation of one of the major contractile proteins, the α-MHC, *in vivo* as well as *in vitro* in an estrogen receptor-dependent mechanism. Furthermore it was shown that estrogen-treatment resulted in an induction of the α-MHC promoter in isolated cardiac myocytes, which was specifically inhibited by the estrogen receptor antagonist, ICI 182780. An Egr-1 mediated pathway was identified as potential pathway involved in the upregulation of α-MHC by estrogen. In the second part of the study the rapid induction of Egr-1 by estrogen via the SREs in the promoter, as previously identified, was further investigated and it was shown, Egr-1 was induced by estrogen via the ERK1/2 pathway and that SRF was recruited to the SREs upon estrogen treatment. It was also shown, that a heterologous promoter containing only 5 SREs in tandem was induced by estrogen in an estrogen receptor-dependent manner, showing that SREs are important promoter elements in the rapid induction of target genes by estrogen. In a third part of the study, an initiation of the search for novel estrogen target genes, identified 24 potentially new targets in the myocardium.

Estrogen has been demonstrated to play a protective role in the heart in numerous studies using animal models (Fraser et al., 2000; Lee et al., 2000) as well as in observations in humans (Gordon et al., 1978; Ho et al., 1993). These studies have mainly concentrated on the vascular system, trying to identify the protective mechanisms induced or modified by estrogen. The Heart and Estrogen/progestin Replacement Study (HERS) was the first prospective and randomised clinical trial on secondary prevention of coronary heart disease by estrogen and progesterone in postmenopausal women. The outcome of this study showed that estrogen replacement therapy had an overall null effect on secondary prevention of coronary disease. However, within this overall null effect, a 50% increase in cardiovascular events due to thrombosis during the first year of therapy was observed, with a

possible beneficial effect if the therapy continues for longer. These observations obviously rose some questions and concerns about hormone replacement therapy as potential treatment for cardiovascular disease and once again showed that not nearly enough is known about estrogen actions in the heart. The HERS trial also focussed only on cardiovascular events and did not investigate the myocardium, with the consequence that no information on the effect of estrogen therapy on the myocardium itself is available.

So far, the mechanisms of action of estrogen in cardiomyocytes themselves have not yet been completely understood. It has been shown that estrogen receptors are expressed and functional in the myocardium (Grohe et al., 1997) and that estrogen can modulate the expression of genes in isolated cardiomyocytes (Nuedling et al., 1999), but no real explanation has so far been given for the mechanism of estrogen action in the myocardium itself and how it might influence the function of the heart. The spectrum of genes regulated by estrogen as well as which mechanisms of action are involved in the estrogen receptor-dependent induction of myocardial genes are not known. Many studies also failed to include specific estrogen receptor antagonists, making it difficult to distinguish between estrogen receptor-dependent and –independent effects. In the present study emphasis was placed on the role of estrogen receptors in modulating myocardial gene expression *in vivo* as well as *in vitro*. The focus of the first part of the study was the investigation of the effect of estrogen on the expression of one of the major myocardial specific contractile proteins, the α-myosin heavy chain. In the second part of the study, the mechanisms of rapid estrogen receptor-dependent gene induction were investigated using the early growth response gene-1 as an example of a target gene that is rapidly induced by estrogen in a mechanism independent of the direct binding of estrogen receptors to estrogen response elements. The third part of the study initiated the search for other estrogen target genes in the myocardium in order to create a global picture of the pattern of myocardial gene expression modulated by estrogen.

5.1 *In vivo* and *in vitro* regulation of α-myosin heavy chain expression by estrogen

In this part of the study it was demonstrated that estrogen has a direct effect on the myocardium which is potent enough to upregulate the expression of α-MHC *in vivo* in spontaneously hypertensive rats (SHR) despite increased blood pressure, which usually reduces α-MHC expression through the development of cardiac hypertrophy. Furthermore, the use of a specific estrogen receptor antagonist revealed that this was a specific estrogen receptor-dependent effect since the upregulation of α-MHC was abolished by the antagonist ICI 182780. These results therefore showed that estrogen receptors do play an important role *in vivo* in the myocardium by regulating the expression of target genes, in this case one of the most important myocardial contractile proteins, in an estrogen receptor-dependent manner. This *in vivo* finding was extended to isolated cardiomyocytes, providing evidence that the upregulation of α-MHC expression in SHR was due to a direct action of estrogen via estrogen receptors on the cardiomyocytes themselves and not due to an effect on the vasculature or fibroblast.

5.1.1 Gender-specific α-myosin heavy chain expression

The contractile proteins, of which the α-myosin heavy chain is most abundantly expressed in the normal adult rat heart, are the working elements of the heart responsible for continuous and rhythmic contraction. Contraction is driven by ATP hydrolysis and the ATPase activity (which is situated in the head region of the MHC, see section 1.5.2 for an overview) determines the velocity of contraction. Since α-MHC has a three times higher ATPase activity than β-MHC (Alpert and Mulieri, 1982; Holubarsch et al., 1985), hearts expressing more α-MHC have a higher contraction velocity than hearts expressing more β-MHC. The latter is associated with slower force generation but more economical contraction in terms of energy consumption. Cardiac hypertrophy and heart failure are associated with decreased α-MHC expression and a reciprocal increase in β-MHC expression (Lompre et al., 1979; Nadal-

Ginard and Mahdavi, 1989) and it has been postulated that the reduction in contraction of the failing myocardium is the result of the reduced amount of α-MHC (Miyata et al., 2000). This postulation was supported by a study where mice with 25% reduced α-MHC content (heterozygous α-MHC knock-out animals) showed decreased systolic function and a characteristic cardiomyopathy with sarcomeric alterations and hypertrophy (Jones et al., 1996). Upregulation of α-MHC expression in the myocardium might therefore contribute to a better contractility of the heart. This was in fact the observation in one hypothyroid patient with left ventricular dysfunction where thyroid replacement resulted in an increase in α-MHC mRNA as well as an increase in left ventricular function (Ladenson et al., 1992). Therefore, the estrogen receptor-dependent upregulation of α-MHC expression in cardiomyocytes investigated in this thesis might contribute to a better contraction efficiency of the heart, at least in rodents. The situation in the human heart is more complex since it contains mostly β-MHC, but our findings can still be seen as paradigmatic and conducive to novel hypotheses in the treatment of heart disease.

The relevance of the estrogen-mediated upregulation of α-MHC and a postulated improvement in cardiac function is supported by gender specific observations in *in vivo* animal studies as well as by *in vitro* analyses of muscle contractility. An *in vivo* study by Douglas et. al. where aortic banding was performed in normotensive Wistar rats showed that the transition to cardiac failure occurred earlier in male than in female rats (Douglas et al., 1998). In another study performed in spontaneously hypertensive rats, it was observed that the development of cardiac failure was earlier in male than in female rats (Tamura et al., 1999).

An association between estrogen and α-MHC expression has been indicated in earlier studies. Malhotra and co-workers used gonadectomised animals exposed to hypertrophic stimuli. All animals developed cardiac hypertrophy, but estrogen replacement attenuated the decrease in α-MHC in both normotensive as well as hypertensive animals (Malhotra et al.,

1990). This result indicated that estrogen induces the expression of α-MHC independently of cardiac hypertrophy, but no further *in vitro* data were presented to support such a theory, nor was a specific estrogen receptor antagonist employed in the study. Two previous reports from the same group also showed that gonadectomy, which was associated with a reduction in α-MHC content and an increase in β-MHC content, also resulted in a decease in cardiac function and a reduction of the mean force-velocity as shown in a working heart model (Schaible et al., 1984; Scheuer et al., 1987). These results supported the hypothesis that estrogen might play a role in improving cardiac function by positively influencing the expression of α-MHC and shifting the α-/β-MHC ratios towards an increased content of α-MHC. In a study from Calovini et. al. the authors showed that ovarectomy resulted in a decrease in α-MHC expression, which was normalised by both estrogen as well as testosterone (Calovini et al., 1995). Again, no judgement can be made on the specificity of the α-MHC regulation by estrogen or testosterone, since neither specific receptor antagonists were not employed in the study, nor were mechanistic data provided. This study indicated that both estrogen as well as testosterone can attenuate the downregulation of α-MHC by ovarectomy. Since aromatases are present and active in the heart (Harada et al., 1999; Price et al., 1992), it might be possible that the supplemented testosterone is aromatised in the heart to produce estrogen, which then influenced the expression of α-MHC.

The strengths of the study presented in this thesis are first of all the inclusion of a specific estrogen receptor antagonist, which made it possible to evaluate the specificity of estrogen action via functional estrogen receptors in the myocardium. The estrogen receptor antagonist data clearly showed that the upregulation of α-MHC by estrogen in the rat heart was due to a direct and specific effect of estrogen receptors on the myocardium and not due to secondary effects such as blood pressure modifications. Secondly, both mRNA as well as protein data are included to extensively investigate this phenomenon. This inclusion of protein data is necessary, since changes in mRNA expression are not always reflected in the protein expression as demonstrated in a study where a dissociation in β-MHC mRNA and protein

expression were found after ascending aortic stenosis in rats (Wiesner et al., 1997). The data presented in this thesis, however, did show a correlation between mRNA and protein data. A third strength of the study is the *in vitro* analyses in isolated cardiomyocytes treated with estrogen alone or in combination with the estrogen receptor antagonist ICI 182780. The *in vitro* data strongly support the *in vivo* data, clearly showing a direct estrogen receptor-dependent mechanism of α-MHC regulation by estrogen in the myocardium. In fact, it was even further characterised by showing that estrogen-treatment resulted in the upregulation of α-MHC promoter activity, which was inhibited by the estrogen receptor antagonist. A fourth strength of the study is data to the mechanisms of α-MHC upregulation by estrogen via an Egr-1-mediated pathway. Egr-1 is rapidly and strongly induced by estrogen in cardiac myocytes and can therefore serve as a key role player in the attenuation of target genes such as α-MHC by estrogen. A fifth strength of the study is the length of estrogen-treatment. Animals were treated over a period of 3 months, making it an investigation into the long term effects of estrogen compared to shorter time points of other studies. These results therefore are all in line with each other, providing for the first time extensive evidence that estrogen directly acts on the myocardium resulting in an attenuation of MHC expression, which might theoretically result in an improvement of cardiac function.

5.1.2 Involvement of ERα and/or ERβ?

Since both estrogen receptor subtypes are present in the heart, it will be interesting to investigate whether ERα and ERβ exert different effects on α-MHC expression. However, the currently available pure estrogen receptor antagonist ICI 182780 binds with similar affinities to both ERα and ERβ (Nicholson et al., 1995). A binding study has already indicated that ICI 182780 might have a higher binding affinity for ERα than for ERβ when used in lower concentrations, but in the concentration used in the current study we assume that both estrogen receptor subtypes were antagonised by ICI 182780. No conclusion can therefore be drawn about the involvement of ERα or ERβ and if there is a difference in effects resulting from the respective receptor subtypes. One way to investigate the roles of the two receptor

subtypes on myocardial gene expression is the use of specific ERα and ERβ agonists. Our group has therefore initiated a follow-up study using specific ERα and ERβ agonists developed by the pharmaceutical industry. In a similar manner, the effects of the agonists on α-MHC expression will be evaluated and the results will shed light on differential effects, if any, resulting from ERα and ERβ action.

5.1.3 Mechanism of α-MHC induction by estrogen

Analysis of the potential mechanism involved in the estrogen receptor-dependent induction of α-MHC showed that estrogen directly induces the α-MHC promoter and that a possible Egr-1 mediated pathway might be involved. First experiments to clarify this hypothesis showed that co-transfection of Egr-1 cDNA together with the α-MHC promoter resulted in an upregulation of α-MHC promoter activity. One previous study by Gupta et. al. showed that the transient overexpression of Egr-1 cDNA in isolated rat cardiomyocytes resulted in an upregulation of α-MHC expression (Gupta et al., 1991), which is in line with our findings. No intact estrogen response elements have been identified in the α-MHC promoter, which makes direct binding of estrogen receptors to the α-MHC promoter less likely. From the results of the present study it is therefore postulated that Egr-1 is rapidly upregulated by estrogen, resulting in increased amounts of Egr-1 protein available to bind to the α-MHC promoter and thereby upregulating its expression. This is unlikely to be the only mechanism involved in the upregulation of α-MHC by estrogen, since the promoter is very large and various other transcription factors are known to regulate its expression (Molkentin et al., 1996) The effect of estrogen on the expression and activity of these other transcription factors has not been investigated, but is an extremely interesting area for further study. It should also be noted that serum response elements are present in the α-MHC promoter. In another part of this study it is shown that estrogen can mediate the expression of target genes in an estrogen receptor-dependent manner via serum response elements. This mechanism has been shown to be involved in the rapid induction of Egr-1 by estrogen and it is shown to be mediated via

ERK1/2. The involvement of SREs in the induction of α-MHC by estrogen has however not yet been investigated and is another area for further research.

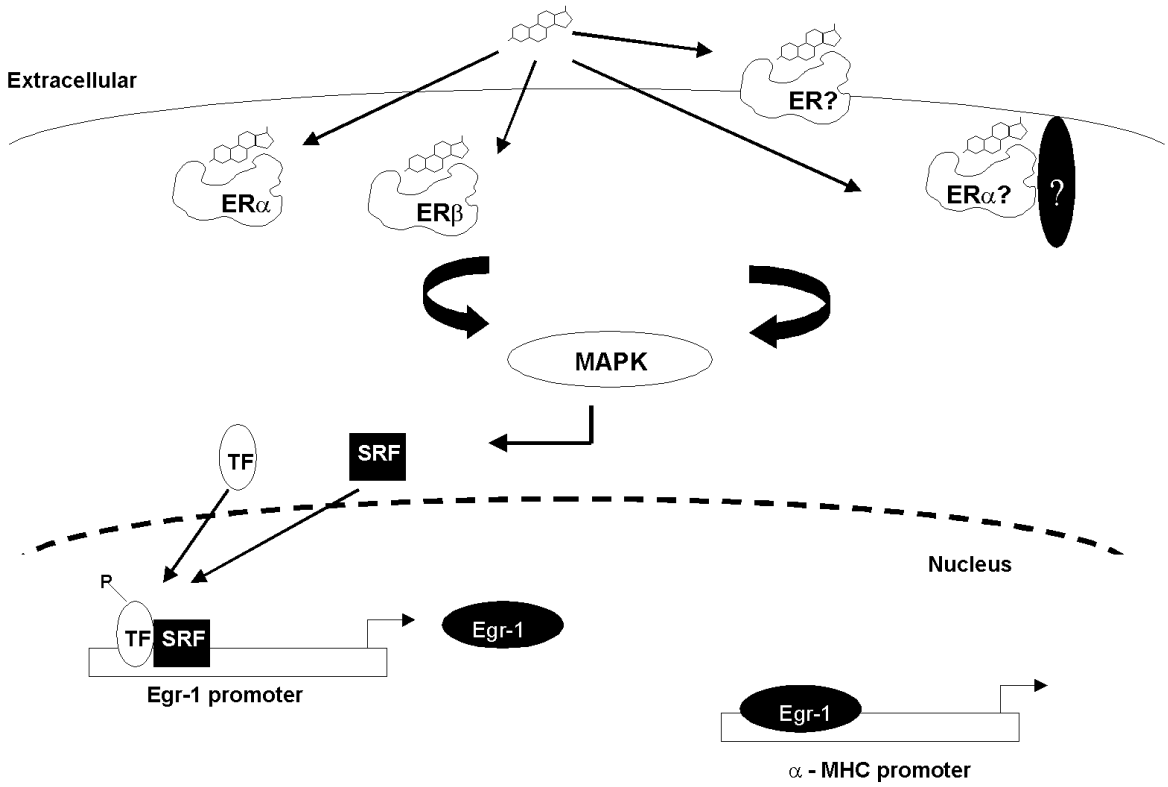


Figure 26: Hypothesis for α - MHC induction by estrogen via an Egr-1 mediated pathway.

Egr-1 is rapidly activated in an estrogen receptor-dependent manner. Estrogen binds to either cytosolic estrogen receptors -α and/or -β, or to a membrane-bound or membrane-associated estrogen receptor. In a manner currently not exactly clear, binding of estrogen to estrogen receptors leads to the downstream activation of the mitogen activated protein kinases (MAPK) ERK1/2, which leads to the recruitment of SRF alone or in combination with an activated transcription factor (TF) probably of the ternary complex factor family to the serum response elements in the Egr-1 promoter. Egr-1 promoter activity is upregulated and Egr-1 protein is available which binds to the Egr-1 binding site in the α-MHC promoter.

5.2 Rapid estrogen effects in cardiomyocytes and mechanisms of target gene induction.

In this part of the study it was shown that estrogen rapidly and transiently induced the phosphorylation of ERK1/2 as well as p38 MAPK in isolated primary neonatal cardiomyocytes. Furthermore, investigations of the mechanisms involved in the rapid

induction of an estrogen target gene (Shamim, 1998) were performed. It was demonstrated that involves binding of the serum response factor or an antigenically related factor to the serum response elements in the Egr-1 promoter. SREs alone can confer an estrogen response to a heterologous promoter, as shown by transfection experiments with an SRE-luc construct, therefore providing more evidence that the SREs in the Egr-1 promoter are responsible for the estrogen response. Together these results identified a novel rapid mechanism of gene activation by estrogen in the myocardium.

5.2.1 ERK1/2 and p38 MAPK activation by estrogen

Estrogen-treatment of primary neonatal cardiomyocytes resulted in the rapid and strong activation of both ERK1/2 as well as the p38 MAPK, showing that estrogen exerts rapid effects in cardiomyocytes by a cross-talk with the signalling pathways of the cells. Nuedling et. al. showed differential effects on the activation of ERK1/2 and p38 MAPK in isolated cardiomyocytes (Nuedling et al., 1999). According to their results, ERK1/2 were rapidly and strongly phosphorylated by estrogen in neonatal as well as adult cardiomyocytes, whereas p38 MAPK was not phosphorylated. However, a slight increase in p38 MAPK activity could be observed as shown in a kinase assay. The different results obtained might be explained by the use of adult cardiomyocytes in their study and neonatal cardiomyocytes in this study.

5.2.2 Target gene induction by estrogen is mediated via SREs in an estrogen receptor-dependent manner.

So far research has focussed on the upstream events leading to the activation of ERK1/2 by estrogen. In this study the focus has been on the downstream events resulting from the activation of ERK1/2. In a previous study in our group, it was shown that Egr-1 was rapidly and transiently activated by estrogen in cardiomyocytes. The Egr-1 promoter contains 2 estrogen response element half-sites (ERE) and 2 AP-1 sites in the distal region and a proximal region with 5 serum response elements. In transfection experiments it was shown that, contrary to the original hypothesis, the ERE/AP-1 sites did not play a role in the

induction of the Egr-1 promoter by estrogen. It was shown that a promoter deletion construct containing the 3 most distal serum response elements in the Egr-1 promoter was significantly induced by estrogen in cardiomyocytes. It was investigated further whether SREs alone were able to confer an estrogen response to a heterologous promoter, by transfection of a construct consisting of 5 SREs linked to luciferase. Estrogen treatment resulted in a 2.3-fold induction of this construct showing that SREs alone are responsive to estrogen. Another study has provided indirect evidence for the involvement of SREs and AP-1 sites in the activation of c-fos by estrogen via MAPK in neuroblastoma cells (Watters et al., 1997), but no direct experiments for the induction via SREs were shown. Recently another study has been published, showing that estrogen induced the c-fos promoter via the SRE (the c-fos promoter contains only one SRE and no EREs) in MCF-7 breast cancer cells (Duan et al., 2001). The authors showed that the induction was mediated in an estrogen receptor-dependent manner via ERK1/2. This study in mammary carcinoma cells was therefore in accordance with our present work in cardiomyocytes, showing that the estrogen receptor mediated gene induction via SREs may also be a rather general phenomenon that can be extrapolated to other target genes and cell types. Further analyses of the protein complexes recruited to the SREs in the Egr-1 promoter upon estrogen-treatment revealed that SRF or an antigenically related protein is recruited to the 3 distal SREs in the Egr-1 promoter. Mutation of the SRE sequence abolished the formation of the protein-DNA complex. Since transcription factors of the ternary complex factor (TCF) family are phosphorylated mainly by ERK1/2 and have also been shown to directly interact with SRF, it cannot be excluded that in addition to SRF a TCF member is involved in the induction of Egr-1 by estrogen. The SREs in the Egr-1 promoter have been implicated as a point of convergence of different signalling pathways activated upon various stimuli in the cell. A detailed analysis of the Egr-1 promoter in lymphatic B-cells, identified the SREs 4 and 5 as crucial for the activation by a mitogenic stimulus (McMahon and Monroe, 1995). Bernal-Mizrachi et. al. showed that Egr-1 is induced upon glucose treatment of pancreatic β-cells via the proximal 2 SREs at least in part in a protein kinase A and Ca^{2+} /CaM dependent manner and not via ERK1/2 (Bernal-Mizrachi et al.,

2000). In the induction of Egr-1 by estrogen, our group has shown that the distal 3 SREs alone are responsive to estrogen and that the induction of Egr-1 is mediated via ERK1/2. These results therefore provide evidence that the SREs in the Egr-1 promoter might act as a point of convergence of different signalling pathways activated upon various stimuli in the cell.

Analysis of the signalling pathways involved in the Egr-1 activation in cardiomyocytes showed that it is specifically mediated via ERK1/2 but not the p38 MAPK pathway. This was demonstrated by the fact that the induction of Egr-1 by estrogen was abolished by the MEK inhibitor PD 98059 but not by the p38 MAPK inhibitor SB 202190. This finding is in accordance with findings in other cell systems showing that Egr-1 activation is mediated by an ERK1/2 dependent mechanism (Chiu et al., 1999; Hodge et al., 1998; Zhang et al., 1998). p38 MAPK has been implicated in the stress-induced activation of Egr-1 in 3T3 fibroblasts (Lim et al., 1998), but this is clearly not the case for the induction of Egr-1 by estrogen in cardiomyocytes.

5.2.3 Relevance of Egr-1 activation in cardiomyocytes

Egr-1 is a transcription factor with an important function in various tissues including the cardiovascular system. In atherosclerotic lesions of mice and humans, elevated levels of Egr-1 mRNA as well as elevated levels of Egr-1 inducible genes such as ICAM-1, PDGF-A, TNFα, TGFβ1, TGFβ3 and SOD1, have been detected implying a function for Egr-1 in repair of vascular lesions after injury (McCaffrey et al., 2000). Furthermore, a function for Egr-1 in the myocardium has been indicated by studies which showed that Egr-1 is highly induced in hypertrophied hearts (Saadane et al., 1999). Our group has shown that Egr-1 can also be induced by hypertrophic stimuli such as endothelin, angiotensin II, adrenoreceptor stimulation or stretch treatment of cardiac myocytes (Neyses et al., 1993; Shamim et al., 1999). A pathophysiologically relevant function for Egr-1 in the myocardium is also suggested by results from Saadane et. al. who showed that the lack of Egr-1 in mice results

in altered expression of pathophysiologically relevant myocardial genes (Saadane et al., 2000). It has also been shown that the overexpression of Egr-1 in neonatal cardiac myocytes resulted in the induction of α-myosin heavy chain, indicating a role of Egr-1 as mediator of myocardial contractile gene expression (Gupta et al., 1991). The induction of Egr-1 by estrogen in the myocardium provides further evidence that estrogen directly acts on cardiac myocytes and represents a possible mechanism by which estrogen could modulate the expression of important myocardial genes downstream of Egr-1 such as the major contractile protein α-myosin heavy chain. In the first part of this study it was shown that estrogen does modulate the expression of α-myosin heavy chain in spontaneously hypertensive rats, an *in vivo* model of cardiac hypertrophy. The *in vivo* findings were furthermore supported and strengthened by *in vitro* data, where it was shown that estrogen-treatment of isolated cardiac myocytes resulted in the upregulation of α-MHC mRNA and protein. The induction of Egr-1 by estrogen might therefore represent a possible mechanism for the alteration of α-myosin heavy chain expression by estrogen.

5.3 Identification of new estrogen target genes in the myocardium

To increase our knowledge on the role of estrogen and estrogen receptors in the myocardium, a search for currently unknown potential estrogen target genes was initiated. So far only a few estrogen responsive genes have been identified in the myocardium, providing insufficient information to create a more global picture of genes regulated by estrogen in general. Knowledge of the spectrum of genes regulated by estrogen might shed some light on the cellular processes influenced by estrogen and estrogen receptors. In breast cancer cells for example, Charpentier and co-workers initiated the search for estrogen responsive genes (Charpentier et al., 2000). This study identified numerous estrogen responsive genes that could be divided into chaperones, cell cycle progression-related genes, paracrine-autocrine factors and tumour-associated genes. These genes were identified by SAGE (serial analysis of gene expression), a technique that allows the

identification of novel genes by the analysis of differentially expressed ESTs (expressed sequence tags) of currently still unknown genes.

For the analysis of potential estrogen target genes in the myocardium, we have used commercially available membrane cDNA array filters with 1250 genes with known function. This method excluded the possibility of identifying novel genes, but has the advantage that the genes investigated have known functions, which facilitates the interpretation and further analysis. A first attempt to try and identify novel estrogen target genes by subtractive cloning did not yield promising results. Subtractive cloning is a very powerful, but stringent technique. We first tested the method performing a test subtraction where cDNA from rat heart was subtracted from a mixed cDNA population from various organs excluding heart. Using this approach, we could identify positive clones only for genes that were highly and specifically expressed in the heart. This result indicated that the technique is suitable for the identification of differentially expressed genes. However, no genes responsive to estrogen could be isolated using this approach, which could be attributed to the relative stringent hybridisation conditions of the subtractive cloning method. The quantitative differences in expression of genes induced by estrogen could be relatively small, such that some genes could have been missed using the subtractive cloning method. Therefore the approach of using cDNA array was chosen. Using this approach it was possible to identify 24 genes that were differentially expressed when mRNA prepared from hearts of ovarectomised animals treated with estrogen were compared to untreated ovarectomised animals. Differentially expressed genes identified by this approach can be divided into transcription factors, ion channels, energy metabolism proteins and ribosomal proteins. The present work together with previous studies in our laboratory have already shown that estrogen induces the transcription factor Egr-1 via the SREs in the promoter region. It was therefore expected that other transcription factors are also modulated by estrogen in rapid, probably non-genomic mechanisms. The transcription factor c-jun contains an AP-1 site in the promoter region

(Cochran, 1993). Since AP- 1 sites have already been shown to confer an estrogen response to genes, it is therefore possible that c-jun is rapidly induced by estrogen via the AP-1 site.

Another interesting group of genes modified by estrogen *in vivo* is the ion channel proteins, which included the KCNE1 gene. Mutations in this gene has been shown to cause long QT syndrome, an inherited cardiac disease associated with arrhythmias and sudden death (Splawski et al., 1997). Gender-specific differences have been observed in long QT syndrome (Lehmann et al., 1997) and it is interesting to speculate that these gender-specific differences in phenotypes might be associated with the regulation of this channel by estrogen in females.

Knowledge on the spectrum of genes potentially modified by estrogen in the myocardium therefore gave some insights into the processes in which estrogen might have an influence *in vivo.* Even though the cDNA microarray analysis is no final proof that estrogen modulates the expression of the identified genes, it can serve as a starting point for further in detail investigations into the role of estrogen in the myocardium. In further studies ongoing in our laboratory, these findings are currently being validated using Northern and Western blotting.

5.4 Future prospects:

This study as well as other studies investigating the role and function of estrogen and estrogen receptors in the myocardium provide detailed information on the different cellular processes and genes influenced by estrogen. Understanding the mechanisms of estrogen action might lead to the identification of targets for future development of better treatment strategies. Certainly it is not feasible to use estrogen as potential treatment for cardiovascular disease, but it serves as a model substance to investigate the positive effects of this hormone. So far the research has already lead to the development of specific ERα and ERβ agonists. Using these substances, it might be possible to specifically target certain genes and tissues without the negative side effects in other tissues which occur with the

natural compounds. Certain phytoestrogens, which are naturally occurring estrogen-like substances in some plants, also have positive effects in certain tissues without the negative side effects in others (Gustafsson, 1998). It is therefore possible to speculate that it will be possible in the future possible to develop optimal estrogen-based treatment strategies for cardiovascular (and potentially other) disease.

Appendix I: Suppliers of materials

17β-estradiol	Sigma
^{32}P γ ATP	ICN
^{32}P-αUTP	Amersham / ICI
Acetic acid	Merck
Agarose	Sigma
Ampicillin	Sigma
Bam HI	Fermentas
Boric Acid	Merck
Bradford protein assay	Biorad
BSA	Sigma
Charcoal stripped FCS	C.C. Pro
CPSR1	Sigma
CTP, TTP ATP, UTP	Roche
DNAse I	Sigma
DTT	Roche
ECL	Amersham
EDTA	Sigma
Estrogen RIA	DPC Bierman
Ethanol	Merck
Formaldehyde	Merck
Formamide	Merck
Glycine	Merck / Serva
Gycerol	Merck
Heparin	Roche
ICI 182780	Tocris
LB-agar	Sigma
L-Glutamine	Sigma

Luciferase Assay	Promega
MEM	Sigma
Methanol	Merck
MOPS	Sigma
NaCl	Merck
Nitrocellulose ECL Membranes	Amersham
OptiMEM	Gibco Life Technologies
Peanut oil	Sigma
Penicillin	Sigma
Peptone	Sigma
pGL3-basic	Promega
Phenol/ Chloroform	Sigma
PIPES	Sigma
Plasmid DNA Maxi and Mini Prep kit	Macheray and Nagal
Polyacrylamide /bisacrylamide solution 30:1	Roth
Poly-dI/dC	Roche
Poly-L-Lysine	Sigma
Proteinase inhibitor cocktail	Roche
Proteinase K	Sigma
pTri-28S-antisense probe	Ambion
Qiaquick gel exrtaction kit	Qiagen
RNAse A	Sigma
RNAse free DNAse I	Roche
RNAse T1	Sigma
RNasin	Promega
Sac I	Fermentas
Schrimp alkaline phosphatase	Roche
SDS	Sigma

Sephadex G50 spin columns	Roche
Streptomycin	Sigma
T_4-DNA ligase	Stratagene
T_7 RNA Polymerase	Promega
Tris base	Merck
Trizol	Gibco Life Technologies
Trypan-Blue	Gibco Life Technologies
Trypsin	Gibco Life Technologies
Urea	Roth
Vitamin B12	Serva
Whattman filter paper	3MM
XI10 Gold Competent Cells	Stratagene
Yeast extract	Gibco Life Technologies
Yeast tRNA	Roche

Appendix II: Buffers and solutions

Cardiomyocytes preparation

CBFHH (Calcium and bicarbonat-free Hanks with Hepes):

8 g NaCl
0.4 g KCl
0.2 g $MgSO_4.7H_2O$
1 g Dextrose
0.06 g KH_2PO_4
0.048 g Na_2HPO_4 anhydrogenous
4.77 g HEPES
H_2O to 1 L
Adjust pH to 7.4

T&D: CBFHH containing 1.5 mg/ml Trypsin and 1 ml DNAse I (2mg/ml in 0.15 M NaCl) pro 100 ml CBFHH

MEM/5 and 2 % CPSR1: To 1L MEM add: 5 ml BrdU (20 mM stock solution)
1 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)
50 ml Charcoal-stripped FCS (for MEM/5)
20 ml CPSR1 (for 2%CPSR1 medium)

RNA

10 x MOPS running buffer: 41.86 g MOPS
6.8 g $NaOAc.3H_2O$
3.8 g Na_4EDTA
H_2O to 1L

Formaldehyde containing Agarose gel for RNA: 1 g Agarose
88 ml H_2O-DEPC
10 ml 10 X MOPS buffer
2.3 µl EtBr
1.87 ml Formaldehyde

RNA loading dye: 720 µl Formamide
160 µl 10 x MOPS buffer

260 µl Formaldehyde
193 µl H_2O
267 µl 6 x Bromophenol blue DNA loading dye

RNase Protection Assay:

10 X PIPES: 60 g PIPES [0.4 M]
116.8 g NaCl [4M]
0.186 g EDTA [10 mM]
Dissolve in DEPC-H_2O in a volume of 500 ml
Adjust pH to 6.7

RNase Cocktail: 300 µl 5M NaCl
50 µl 1M Tris pH 7.5
50 µl 0.5 M EDTA
10 µl RNase A (10mg/ml)
7.5 µl RNase T1
H_2O to 5 ml

6 % Denaturing PAGE: 22 g Urea
20 ml H_2O
5 ml TBE
8.5 ml 30% Acrylamide/0.8% Bis-acrylamide
500 µl APS
50 µl Temed

Loading dye: 50% Formamide
0.05% Bromophenol blue
0.05% Xylene cyanol

Western blotting:

RIPA Buffer: 1 X PBS
1 % Igepal CA-630
0.5 % Sodium deoxycholate
0.1 % SDS

5 X Running buffer: 15 g Tris-Base
72 g Glycin

5 g SDS
H_2O to 1 litre
pH 8.3

Transfer buffer: 200 ml 5 X running buffer
200 ml Methanol
600 ml H_2O

5 X PBS: 41,17 g Na_2HPO_4 (MW =
11,73 g NaH_2PO_4 (MW =
20 g NaCl
H_2O to 1 L
pH 7,4

PBS-T: 1 X PBS with 0,05% Tween 20

Western blot stripping buffer: 31,2 ml 500 mM Tris pH 6.7
50 ml 10% SDS
H_2O to 200 ml

Seperating SDS Gels:

	7,5 %	**10 %**	**15 %**
H_2O	9,7 ml	8 ml	4,7 ml
1.5 M Tris (pH 8.8)	5 ml	5 ml	5 ml
10 % SDS	200 µl	200 µl	200 µl
Acrylamide/Bis (30 %)	5 ml	6,67 ml	10 ml
10 % APS	100 µl	100 µl	100µl
TEMED	10 µl	10 µl	10 µl

5 % Stacking gels:

H_2O	2.85 ml
0.5 M Tris (pH 6.8)	1.25 ml
10 % SDS	100 µl
Acrylamide/Bis (30 %)	850 µl
10 % APS	50 µl
TEMED	5 µl

α - MHC SDS Gelelectrophoresis:

α-MHC Sample Buffer: 0.3 M NaCl
0.1 M NaH_2PO_4
50 mM Na_2HPO_4
10 mM Na Pyrophosphat pH 7.4
1 mM $MgCl_2$
10 mM EDTA

8 X Electrophoresis buffer: 0.2 M Tris pH 8.3
1.54 M Glycine
0.8 % SDS

6 % SDS-Glycine gel containing 5 % glycerol:

	6 % Seperating gel	**4% Stacking gel**
100 % Glycerol	1 ml	0.5 ml
23 % Acrylamide/Bis (50:1)	5.3 ml	1.7 ml
1.5 M Tris (pH 8.8)	2.7 ml	---
0.5 M Tris (pH 6.8)	---	1.4 ml
1 M Glycine	2 ml	---
0.5 M EDTA (pH 8.0)	---	80 µl
10 % SDS	800 µl	400 µl
H_2O	8 ml	5.8 ml
10 % APS	200 µl	100 µl
TEMED	10 µl	5 µl

EMSA:

Buffer A: 10 mM Hepes pH 7.9
10 mM KCl
0.2 mM EGTA
1 mM DTT
0.5 mM PMSF

Buffer C: 20 mM Hepes pH 7.9
0.4 M NaCl
2 mM EGTA
1 mM DTT

1 mM PMSF

3 x Binding buffer: 60 mM Hepes pH 7.8
3 mM DTT
3 mM EGTA
150 mM KCl
12 % Ficoll
1 mM $ZnCl_2$

Electrophoresis Buffers:

10 X TBE: 108 g Tris base
55 g Boric acid
40 ml 0.05 M EDTA pH 8.0
H_2O to 1 L

50 X TAE: 242 g Tris base
57.1 ml Glacial acetic acid
100 ml 0.5 M EDTA

Bacterial growth medium:

LB: 10 g NaCl
5 g Yeast Extract
10 g Peptone
H_2O to 1L

cDNA Array:

10 x DNase I Buffer: 400 mM Tris-HCl (pH 7.5)
100 mM NaCl
60 mM $MgCl_2$

20 x SSC: 175.3 g NaCl
88.2 g $Na_3Citrate.2H_2O$

20% SDS: 200 g SDS
Add H_2O to 1L

Primers for GAPDH amplification:

Forward primer: 5’ – ACC ACA GTC CAT GCC ATC AC – 3’

Reverse primer: 5’ – TCC ACC ACC CTG TTG CTG TA – 3’

Acknowledgements

I would like to thank the following people and institutions for their help and support during the preparation of this thesis:

- My study leader and supervisor, Prof. Dr. Ludwig Neyses, for his helpful and extremely motivating discussions, encouragement and continuos support not only during the time spend in his laboratory, but also thereafter. I have learned a lot during the time in your laboratory.
- Prof. Dr. Klaus Scheller for the supervision of the work in the Department of Biology, University of Würzburg.
- The German Academic Exchange Service (DAAD) for enabling me to come to Germany for my PhD studies by providing the financial support.
- Dr. med. Theo Pelzer for his help and support of the project and preparation of the thesis.
- Jenny Muck for her excellent technical assistance and help in the laboratory. It was a pleasure to work with you.
- Stephan Müller-Botz for his co-operation and input in the project. It was fun to work with you!
- The members of “Lab Neyses” for creating a pleasant working environment and their help in the laboratory.
- Astrid Bischof for dealing with the “frustrating times” as well as her support during the 3 years.
- Meike Pellens for her patience and support during the final preparation of the thesis.
- Last but not least, I would like to thank my family in South Africa for their non-stop support, encouragement and understanding during my PhD years.

Curriculum vitae

Name:	Tertia de Jager
Date of birth:	29.01.1971
Nationality:	South African
High school:	Gymnasium High School, Paarl, South Africa
Matriculation:	1988 (A-average)
Degrees obtained:	1) Bachelors of Science (Biochemistry and Genetics), 1991, University of Stellenbosch 2) Bachelors of Science; Honours degree (Medical Biochemistry), 1992, University of Stellenbosch 3) Masters of Science *cum laude* (Medical Biochemistry), 1994, University of Stellenbosch
Feb. 1995 – Oct. 1995:	Research visit to the laboratory of Dr. Mark Keating, Howard Hughes Medical Institute, University of Utah, Salt Lake City, USA.
Nov. 1995 – June 1997:	Appointed as Junior Scientist at the South African Medical Research Council and University of Stellenbosch
Aug 1997 – Sep 1997:	German language course at the Goethe Institute, Mannheim
Oct. 1997 – April 2001:	PhD studies at the Department of Medicine, University of Würzburg with a stipend from the German academic exchange program (DAAD)
May 2001 – current time:	Postdoctoral position in the Centre for Molecular Neurobiology, Institute of Pathoneurobiology, AG Jentsch

Publications by Tertia de Jager

1) Brink PA, Moolman JC, Ferreira A, De Jager T, Weymar HW, Martell RW, Torrington M, Van der Merwe P-L, Corfield VA. Genetic linkage studies of progressive familial heart block, a cardiac conduction disorder. South African Journal of Science 1994; 90:236 – 240.

2) Wang Q, Curran ME, Splawski I, Burn TC, Milholland JM, VanRaay TJ, Shen J, Timothy KW, Vincent GM, De Jager T, Schwartz PJ, Towbin JA, Moss AJ, Atkinson HL, Landes GM, Connors TD, Keating MT. Positional cloning of a novel potassium channel gene: KVLQT1 mutations cause cardiac arrythmias. Nature Genetics 1996; 12:17 –23.

3) De Jager T, Corbett CH, Badenhorst JCW, Brink PA, Corfield VA. Evidence of a long QT founder gene with varying phenotypic expression in South African families. Journal of Medical Genetics 1996; 33:567 - 573.

4) Pelzer T, Schumann M, Neuman M, De Jager T, Stimpel M, Serfling E, Neyses L. 17ß-Estradiol prevents programmed cell death in cardiac myocytes. Biochemical and Biophysical Research Communications 2000; 268: 192 – 200

5) De Jager T *, Pelzer T *, Mueller-Botz S, Imam A, Muck J, Neyses L. Mechanisms of estrogen receptor action in the myocardium: rapid gene activation via the ERK1/2 pathway and serum response elements. Journal of Biological Chemistry 2001; 276 (30): 27873 – 27880
   * = authors contributed equally to the work

6) Pelzer T, Neumann M, De Jager T, Jazbutyte V, Neyses L. Estrogen effects in the myocardium: Inhibition of NF-κB DNA binding by estrogen. Biochemical and Biophysical Research Communications 2001; 286 (5): 1153 – 1157

7) Pelzer T *, De Jager T *, Muck J, Stimpel M, Neyses L. Estrogen action on the myocardium in vivo: specific and permissive for ACE-inhibition. Journal of Hypertension 2002; 20 (5) in press
   * = authors contributed equally to the work

References

Alpert, N. R., and Mulieri, L. A. (1982). Increased myothermal economy of isometric force generation in compensated cardiac hypertrophy induced by pulmonary artery constriction in the rabbit. A characterization of heat liberation in normal and hypertrophied right ventricular papillary muscles. Circ Res *50*, 491-500.

Barr, D. P., Russ, E. M., and Ha, E. (1952). Iinfluence of oestrogen on lipoproteins in atherosclerosis. Transactions of the Association of American Physicians *65*, 102 - 111.

Barrett-Connor, E., and Bush, T. L. (1991). Estrogen and coronary heart disease in women. Jama *265*, 1861-7.

Bernal-Mizrachi, E., Wice, B., Inoue, H., and Permutt, M. A. (2000). Activation of serum response factor in the depolarization induction of Egr-1 transcription in pancreatic islet beta-cells. J Biol Chem *275*, 25681-9.

Bing, O. H., Brooks, W. W., Robinson, K. G., Slawsky, M. T., Hayes, J. A., Litwin, S. E., Sen, S., and Conrad, C. H. (1995). The spontaneously hypertensive rat as a model of the transition from compensated left ventricular hypertrophy to failure. J Mol Cell Cardiol *27*, 383-96.

Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature *389*, 753-8.

Bush, T. L., Barrett-Connor, E., Cowan, L. D., Criqui, M. H., Wallace, R. B., Suchindran, C. M., Tyroler, H. A., and Rifkind, B. M. (1987). Cardiovascular mortality and noncontraceptive use of estrogen in women: results from the Lipid Research Clinics Program Follow-up Study. Circulation *75*, 1102-9.

Calovini, T., Haase, H., and Morano, I. (1995). Steroid-hormone regulation of myosin subunit expression in smooth and cardiac muscle. J Cell Biochem *59*, 69-78.

Chambliss, K. L., Yuhanna, I. S., Mineo, C., Liu, P., German, Z., Sherman, T. S., Mendelsohn, M. E., Anderson, R. G., and Shaul, P. W. (2000). Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. Circ Res *87*, E44-52.

Chang, W. C., Nakao, J., Orimo, H., and Murota, S. I. (1980). Stimulation of prostaglandin cyclooxygenase and prostacyclin synthetase activities by estradiol in rat aortic smooth muscle cells. Biochim Biophys Acta *620*, 472-82.

Charpentier, A. H., Bednarek, A. K., Daniel, R. L., Hawkins, K. A., Laflin, K. J., Gaddis, S., MacLeod, M. C., and Aldaz, C. M. (2000). Effects of estrogen on global gene expression: identification of novel targets of estrogen action. Cancer Res *60*, 5977-83.

Chiu, J. J., Wung, B. S., Hsieh, H. J., Lo, L. W., and Wang, D. L. (1999). Nitric oxide regulates shear stress-induced early growth response-1. Expression via the extracellular signal-regulated kinase pathway in endothelial cells. Circ Res *85*, 238-46.

Cochran, B. H. (1993). Regulation of immediate early gene expression. NIDA Res Monogr *125*, 3-24.

Collins, P., Rosano, G. M., Jiang, C., Lindsay, D., Sarrel, P. M., and Poole-Wilson, P. A. (1993). Cardiovascular protection by oestrogen--a calcium antagonist effect? Lancet *341*, 1264-5.

Collins, P., Rosano, G. M., Sarrel, P. M., Ulrich, L., Adamopoulos, S., Beale, C. M., McNeill, J. G., and Poole-Wilson, P. A. (1995). 17 beta-Estradiol attenuates acetylcholine-induced coronary arterial constriction in women but not men with coronary heart disease. Circulation *92*, 24-30.

Conrad, C. H., Brooks, W. W., Hayes, J. A., Sen, S., Robinson, K. G., and Bing, O. H. (1995). Myocardial fibrosis and stiffness with hypertrophy and heart failure in the spontaneously hypertensive rat. Circulation *91*, 161-70.

Cummings, S. R., Eckert, S., Krueger, K. A., Grady, D., Powles, T. J., Cauley, J. A., Norton, L., Nickelsen, T., Bjarnason, N. H., Morrow, M., Lippman, M. E., Black, D., Glusman, J. E., Costa, A., and Jordan, V. C. (1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. Jama *281*, 2189-97.

Das, S. K., Taylor, J. A., Korach, K. S., Paria, B. C., Dey, S. K., and Lubahn, D. B. (1997). Estrogenic responses in estrogen receptor-alpha deficient mice reveal a distinct estrogen signaling pathway. Proc Natl Acad Sci U S A *94*, 12786-91.

Davis, C. E., Pajak, A., Rywik, S., Williams, D. H., Broda, G., Pazucha, T., and Ephross, S. (1994). Natural menopause and cardiovascular disease risk factors. The Poland and US Collaborative Study on Cardiovascular Disease Epidemiology. Ann Epidemiol *4*, 445-8.

De Jager, T., Pelzer, T., Mueller-Botz, S., Imam, A., Muck, J., and Neyses, L. (2001). Mechanisms of estrogen receptor action in the myocardium: rapid gene activation via the ERK1/2 pathway and serum response elements. J Biol Chem *276*, 27873-27880.

Douglas, P. S., Katz, S. E., Weinberg, E. O., Chen, M. H., Bishop, S. P., and Lorell, B. H. (1998). Hypertrophic remodeling: gender differences in the early response to left ventricular pressure overload. J Am Coll Cardiol *32*, 1118-25.

Duan, R., Xie, W., Burghardt, R. C., and Safe, S. (2001). Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. J Biol Chem *276*, 11590-8.

Engelmann, G. L., Vitullo, J. C., and Gerrity, R. G. (1987). Morphometric analysis of cardiac hypertrophy during development, maturation, and senescence in spontaneously hypertensive rats. Circ Res *60*, 487-94.

Farhat, M. Y., Lavigne, M. C., and Ramwell, P. W. (1996). The vascular protective effects of estrogen. Faseb J *10*, 615-24.

Fraser, H., Davidge, S. T., and Clanachan, A. S. (2000). Activation of Ca(2+)-independent nitric oxide synthase by 17beta- estradiol in post-ischemic rat heart. Cardiovasc Res *46*, 111-8.

Freay, A. D., Curtis, S. W., Korach, K. S., and Rubanyi, G. M. (1997). Mechanism of vascular smooth muscle relaxation by estrogen in depolarized rat and mouse aorta. Role of nuclear estrogen receptor and Ca2+ uptake. Circ Res *81*, 242-8.

Gilligan, D. M., Badar, D. M., Panza, J. A., Quyyumi, A. A., and Cannon, R. O., 3rd (1994). Acute vascular effects of estrogen in postmenopausal women. Circulation *90*, 786-91.

Gilligan, D. M., Quyyumi, A. A., and Cannon, R. O., 3rd (1994). Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women. Circulation *89*, 2545-51.

Gisclard, V., Miller, V. M., and Vanhoutte, P. M. (1988). Effect of 17 beta-estradiol on endothelium-dependent responses in the rabbit. J Pharmacol Exp Ther *244*, 19-22.

Gordon, T., Kannel, W. B., Hjortland, M. C., and McNamara, P. M. (1978). Menopause and coronary heart disease. The Framingham Study. Ann Intern Med *89*, 157-61.

Grady, D., Rubin, S. M., Petitti, D. B., Fox, C. S., Black, D., Ettinger, B., Ernster, V. L., and Cummings, S. R. (1992). Hormone therapy to prevent disease and prolong life in postmenopausal women. Ann Intern Med *117*, 1016-37.

Green, S., Walter, P., Greene, G., Krust, A., Goffin, C., Jensen, E., Scrace, G., Waterfield, M., and Chambon, P. (1986). Cloning of the human oestrogen receptor cDNA. J Steroid Biochem *24*, 77-83.

Grodstein, F., Stampfer, M. J., Goldhaber, S. Z., Manson, J. E., Colditz, G. A., Speizer, F. E., Willett, W. C., and Hennekens, C. H. (1996). Prospective study of exogenous hormones and risk of pulmonary embolism in women. Lancet *348*, 983-7.

Grohe, C., Kahlert, S., Lobbert, K., Meyer, R., Linz, K. W., Karas, R. H., and Vetter, H. (1996). Modulation of hypertensive heart disease by estrogen. Steroids *61*, 201-4.

Grohe, C., Kahlert, S., Lobbert, K., Stimpel, M., Karas, R. H., Vetter, H., and Neyses, L. (1997). Cardiac myocytes and fibroblasts contain functional estrogen receptors. FEBS Lett *416*, 107-12.

Guetta, V., Quyyumi, A. A., Prasad, A., Panza, J. A., Waclawiw, M., and Cannon, R. O., 3rd (1997). The role of nitric oxide in coronary vascular effects of estrogen in postmenopausal women. Circulation *96*, 2795-801.

Gupta, M. P., Gupta, M., Zak, R., and Sukhatme, V. P. (1991). Egr-1, a serum-inducible zinc finger protein, regulates transcription of the rat cardiac alpha-myosin heavy chain gene. J Biol Chem *266*, 12813-6.

Gustafsson, J. A. (1998). Therapeutic potential of selective estrogen receptor modulators. Cur Opin Chem Biol *2*, 508-511.

Harada, N., Sasano, H., Murakami, H., Ohkuma, T., Nagura, H., and Takagi, Y. (1999). Localized expression of aromatase in human vascular tissues. Circ Res *84*, 1285-91.

Harder, D. R., and Coulson, P. B. (1979). Estrogen receptors and effects of estrogen on membrane electrical properties of coronary vascular smooth muscle. J Cell Physiol *100*, 375-82.

Hayashi, T., Fukuto, J. M., Ignarro, L. J., and Chaudhuri, G. (1992). Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: implications for atherosclerosis. Proc Natl Acad Sci U S A *89*, 11259-63.

Hayward, C. S., Kelly, R. P., and Collins, P. (2000). The roles of gender, the menopause and hormone replacement on cardiovascular function. Cardiovasc Res *46*, 28-49.

Herrington, D. M. (1999). The HERS trial results: paradigms lost? Heart and Estrogen/progestin Replacement Study. Ann Intern Med *131*, 463-6.

Ho, K. K., Anderson, K. M., Kannel, W. B., Grossman, W., and Levy, D. (1993). Survival after the onset of congestive heart failure in Framingham Heart Study subjects. Circulation *88*, 107-15.

Hodge, C., Liao, J., Stofega, M., Guan, K., Carter-Su, C., and Schwartz, J. (1998). Growth hormone stimulates phosphorylation and activation of elk-1 and expression of c-fos, egr-1, and junB through activation of extracellular signal-regulated kinases 1 and 2. J Biol Chem *273*, 31327-36.

Holubarsch, C., Goulette, R. P., Litten, R. Z., Martin, B. J., Mulieri, L. A., and Alpert, N. R. (1985). The economy of isometric force development, myosin isoenzyme pattern and myofibrillar ATPase activity in normal and hypothyroid rat myocardium. Circ Res *56*, 78-86.

Improta-Brears, T., Whorton, A. R., Codazzi, F., York, J. D., Meyer, T., and McDonnell, D. P. (1999). Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc Natl Acad Sci U S A *96*, 4686-91.

Jiang, C., Poole-Wilson, P. A., Sarrel, P. M., Mochizuki, S., Collins, P., and MacLeod, K. T. (1992). Effect of 17 beta-oestradiol on contraction, Ca2+ current and intracellular free Ca2+ in guinea-pig isolated cardiac myocytes. Br J Pharmacol *106*, 739-45.

Jiang, C., Sarrel, P. M., Poole-Wilson, P. A., and Collins, P. (1992). Acute effect of 17 beta-estradiol on rabbit coronary artery contractile responses to endothelin-1. Am J Physiol *263*, H271-5.

Johnson, B. D., Zheng, W., Korach, K. S., Scheuer, T., Catterall, W. A., and Rubanyi, G. M. (1997). Increased expression of the cardiac L-type calcium channel in estrogen receptor-deficient mice. J Gen Physiol *110*, 135-40.

Jones, W. K., Grupp, I. L., Doetschman, T., Grupp, G., Osinska, H., Hewett, T. E., Boivin, G., Gulick, J., Ng, W. A., and Robbins, J. (1996). Ablation of the murine alpha myosin heavy chain gene leads to dosage effects and functional deficits in the heart. J Clin Invest *98*, 1906-17.

Kannel, W. B., Hjortland, M. C., McNamara, P. M., and Gordon, T. (1976). Menopause and risk of cardiovascular disease: the Framingham study. Ann Intern Med *85*, 447-52.

Karas, R. H., Baur, W. E., van Eickles, M., and Mendelsohn, M. E. (1995). Human vascular smooth muscle cells express an estrogen receptor isoform. FEBS Lett *377*, 103-8.

Kato, S., Tora, L., Yamauchi, J., Masushige, S., Bellard, M., and Chambon, P. (1992). A far upstream estrogen response element of the ovalbumin gene contains several half-palindromic 5'-TGACC-3' motifs acting synergistically. Cell *68*, 731-42.

Keaney, J. F., Jr., Shwaery, G. T., Xu, A., Nicolosi, R. J., Loscalzo, J., Foxall, T. L., and Vita, J. A. (1994). 17 beta-estradiol preserves endothelial vasodilator function and limits low-density lipoprotein oxidation in hypercholesterolemic swine. Circulation *89*, 2251-9.

Kim, H. P., Lee, J. Y., Jeong, J. K., Bae, S. W., Lee, H. K., and Jo, I. (1999). Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. Biochem Biophys Res Commun *263*, 257-62.

Koh, K. K., Mincemoyer, R., Bui, M. N., Csako, G., Pucino, F., Guetta, V., Waclawiw, M., and Cannon, R. O., 3rd (1997). Effects of hormone-replacement therapy on fibrinolysis in postmenopausal women. N Engl J Med *336*, 683-90.

Korach, K. S., Couse, J. F., Curtis, S. W., Washburn, T. F., Lindzey, J., Kimbro, K. S., Eddy, E. M., Migliaccio, S., Snedeker, S. M., Lubahn, D. B., Schomberg, D. W., and Smith, E. P. (1996). Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. Recent Prog Horm Res *51*, 159-86.

Kuch, B., Hense, H., and Muscholl, M. (1997). Postmenopausal hormone replacement therapy and left ventricular mass and geometry: J Am coll Cardiol), pp. 297A.

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J. A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology *138*, 863-70.

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A *93*, 5925-30.

Ladenson, P. W., Sherman, S. I., Baughman, K. L., Ray, P. E., and Feldman, A. M. (1992). Reversible alterations in myocardial gene expression in a young man with dilated cardiomyopathy and hypothyroidism. Proc Natl Acad Sci U S A *89*, 5251-5.

Lee, H. W., and Eghbali-Webb, M. (1998). Estrogen enhances proliferative capacity of cardiac fibroblasts by estrogen receptor- and mitogen-activated protein kinase-dependent pathways. J Mol Cell Cardiol *30*, 1359-68.

Lee, T. M., Su, S. F., Tsai, C. C., Lee, Y. T., and Tsai, C. H. (2000). Cardioprotective effects of 17 beta-estradiol produced by activation ofmitochondrial ATP-sensitive K(+)Channels in canine hearts. J Mol Cell Cardiol *32*, 1147-58.

Lehmann, M. H., Timothy, K. W., Frankovich, D., Fromm, B. S., Keating, M., Locati, E. H., Taggart, R. T., Towbin, J. A., Moss, A. J., Schwartz, P. J., and Vincent, G. M. (1997). Age-gender influence on the rate-corrected QT interval and the QT-heart rate relation in families with genotypically characterized long QT syndrome. J Am Coll Cardiol *29*, 93-9.

Levy, D., Larson, M. G., Vasan, R. S., Kannel, W. B., and Ho, K. K. (1996). The progression from hypertension to congestive heart failure. Jama *275*, 1557-62.

Li, Z., Bing, O. H., Long, X., Robinson, K. G., and Lakatta, E. G. (1997). Increased cardiomyocyte apoptosis during the transition to heart failure in the spontaneously hypertensive rat. Am J Physiol *272*, H2313-9.

Lieberman, E. H., Gerhard, M. D., Uehata, A., Walsh, B. W., Selwyn, A. P., Ganz, P., Yeung, A. C., and Creager, M. A. (1994). Estrogen improves endothelium-dependent, flow-mediated vasodilation in postmenopausal women. Ann Intern Med *121*, 936-41.

Lim, C. P., Jain, N., and Cao, X. (1998). Stress-induced immediate-early gene, egr-1, involves activation of p38/JNK1. Oncogene *16*, 2915-26.

Lim, W. K., Wren, B., Jepson, N., Roy, S., and Caplan, G. (1999). Effect of hormone replacement therapy on left ventricular hypertrophy. Am J Cardiol *83*, 1132-4, A9.

Lompre, A. M., Schwartz, K., d'Albis, A., Lacombe, G., Van Thiem, N., and Swynghedauw, B. (1979). Myosin isoenzyme redistribution in chronic heart overload. Nature *282*, 105-7.

Lowes, B. D., Minobe, W., Abraham, W. T., Rizeq, M. N., Bohlmeyer, T. J., Quaife, R. A., Roden, R. L., Dutcher, D. L., Robertson, A. D., Voelkel, N. F., Badesch, D. B., Groves, B. M., Gilbert, E. M., and Bristow, M. R. (1997). Changes in gene expression in the intact human heart. Downregulation of alpha-myosin heavy chain in hypertrophied, failing ventricular myocardium. J Clin Invest *100*, 2315-24.

Magness, R. R., and Rosenfeld, C. R. (1989). Local and systemic estradiol-17 beta: effects on uterine and systemic vasodilation. Am J Physiol *256*, E536-42.

Malhotra, A., Buttrick, P., and Scheuer, J. (1990). Effects of sex hormones on development of physiological and pathological cardiac hypertrophy in male and female rats. Am J Physiol *259*, H866-71.

Marsh, J. D., Lehmann, M. H., Ritchie, R. H., Gwathmey, J. K., Green, G. E., and Schiebinger, R. J. (1998). Androgen receptors mediate hypertrophy in cardiac myocytes. Circulation *98*, 256-61.

Mashiah, A., Berman, V., Thole, H. H., Rose, S. S., Pasik, S., Schwarz, H., and Ben-Hur, H. (1999). Estrogen and progesterone receptors in normal and varicose saphenous veins. Cardiovasc Surg *7*, 327-31.

Matthews, K. A., Meilahn, E., Kuller, L. H., Kelsey, S. F., Caggiula, A. W., and Wing, R. R. (1989). Menopause and risk factors for coronary heart disease. N Engl J Med *321*, 641-6.

McCaffrey, T. A., Fu, C., Du, B., Eksinar, S., Kent, K. C., Bush, H., Jr., Kreiger, K., Rosengart, T., Cybulsky, M. I., Silverman, E. S., and Collins, T. (2000). High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. J Clin Invest *105*, 653-62.

McCrohon, J. A., Adams, M. R., McCredie, R. J., Robinson, J., Pike, A., Abbey, M., Keech, A. C., and Celermajer, D. S. (1996). Hormone replacement therapy is associated with improved arterial physiology in healthy post-menopausal women. Clin Endocrinol (Oxf) *45*, 435-41.

McMahon, S. B., and Monroe, J. G. (1995). A ternary complex factor-dependent mechanism mediates induction of egr- 1 through selective serum response elements following antigen receptor cross-linking in B lymphocytes. Mol Cell Biol *15*, 1086-93.

Mendelsohn, M. E., and Karas, R. H. (1994). Estrogen and the blood vessel wall. Curr Opin Cardiol *9*, 619-26.

Mendelsohn, M. E., and Karas, R. H. (1999). The protective effects of estrogen on the cardiovascular system. N Engl J Med *340*, 1801-11.

Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996). Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. Embo J *15*, 1292-300.

Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. (1998). Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. Embo J *17*, 2008-18.

Mitlak, B. H., and Cohen, F. J. (1997). In search of optimal long-term female hormone replacement: the potential of selective estrogen receptor modulators. Horm Res *48*, 155-63.

Miyata, S., Minobe, W., Bristow, M. R., and Leinwand, L. A. (2000). Myosin heavy chain isoform expression in the failing and nonfailing human heart. Circ Res *86*, 386-90.

Molkentin, J. D., Jobe, S. M., and Markham, B. E. (1996). Alpha-myosin heavy chain gene regulation: delineation and characterization of the cardiac muscle-specific enhancer and muscle- specific promoter. J Mol Cell Cardiol *28*, 1211-25.

Mugge, A., Riedel, M., Barton, M., Kuhn, M., and Lichtlen, P. R. (1993). Endothelium independent relaxation of human coronary arteries by 17 beta-oestradiol in vitro. Cardiovasc Res *27*, 1939-42.

Nadal, A., Ropero, A. B., Laribi, O., Maillet, M., Fuentes, E., and Soria, B. (2000). Nongenomic actions of estrogens and xenoestrogens by binding at a plasma membrane receptor unrelated to estrogen receptor alpha and estrogen receptor beta [In Process Citation]. Proc Natl Acad Sci U S A *97*, 11603-8.

Nadal, A., Rovira, J. M., Laribi, O., Leon-quinto, T., Andreu, E., Ripoll, C., and Soria, B. (1998). Rapid insulinotropic effect of 17beta-estradiol via a plasma membrane receptor. Faseb J *12*, 1341-8.

Nadal-Ginard, B., and Mahdavi, V. (1989). Molecular basis of cardiac performance. Plasticity of the myocardium generated through protein isoform switches. J Clin Invest *84*, 1693-700.

Nakao, K., Minobe, W., Roden, R., Bristow, M. R., and Leinwand, L. A. (1997). Myosin heavy chain gene expression in human heart failure. J Clin Invest *100*, 2362-70.

Neyses, L., Nouskas, J., Luyken, J., Fronhoffs, S., Oberdorf, S., Pfeifer, U., Williams, R. S., Sukhatme, V. P., and Vetter, H. (1993). Induction of immediate-early genes by angiotensin II and endothelin-1 in adult rat cardiomyocytes. J Hypertens *11*, 927-34.

Nicholson, R. I., Gee, J. M., Manning, D. L., Wakeling, A. E., Montano, M. M., and Katzenellenbogen, B. S. (1995). Responses to pure antiestrogens (ICI 164384, ICI 182780) in estrogen- sensitive and -resistant experimental and clinical breast cancer. Ann N Y Acad Sci *761*, 148-63.

Norfleet, A. M., Clarke, C. H., Gametchu, B., and Watson, C. S. (2000). Antibodies to the estrogen receptor-alpha modulate rapid prolactin release from rat pituitary tumor cells through plasma membrane estrogen receptors. Faseb J *14*, 157-65.

Nuedling, S., Kahlert, S., Loebbert, K., Doevendans, P. A., Meyer, R., Vetter, H., and Grohe, C. (1999). 17 Beta-estradiol stimulates expression of endothelial and inducible NO synthase in rat myocardium in-vitro and in-vivo. Cardiovasc Res *43*, 666-74.

Nuedling, S., Kahlert, S., Loebbert, K., Meyer, R., Vetter, H., and Grohe, C. (1999). Differential effects of 17beta-estradiol on mitogen-activated protein kinase pathways in rat cardiomyocytes. FEBS Lett *454*, 271-6.

Opie, L. H. (1998). The Heart: Physiology, from cell to circulation, 3 Edition: Lippincott-Raven Publishers).

Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J., and Scanlan, T. S. (1997). Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science *277*, 1508-10.

Patterson, E., Ma, L., Szabo, B., Robinson, C. P., and Thadani, U. (1998). Ovariectomy and estrogen-induced alterations in myocardial contractility in female rabbits: role of the L-type calcium channel. J Pharmacol Exp Ther *284*, 586-91.

Pelzer, T., Schumann, M., Neumann, M., deJager, T., Stimpel, M., Serfling, E., and Neyses, L. (2000). 17beta-estradiol prevents programmed cell death in cardiac myocytes. Biochem Biophys Res Commun *268*, 192-200.

Pfeffer, J. M., Pfeffer, M. A., Fishbein, M. C., and Frohlich, E. D. (1979). Cardiac function and morphology with aging in the spontaneously hypertensive rat. Am J Physiol *237*, H461-8.

Pines, A., Fisman, E. Z., Levo, Y., Averbuch, M., Lidor, A., Drory, Y., Finkelstein, A., Hetman-Peri, M., Moshkowitz, M., Ben-Ari, E., and et al. (1991). The effects of hormone replacement therapy in normal postmenopausal women: measurements of Doppler-derived parameters of aortic flow. Am J Obstet Gynecol *164*, 806-12.

Price, T., Aitken, J., and Simpson, E. R. (1992). Relative expression of aromatase cytochrome P450 in human fetal tissues as determined by competitive polymerase chain reaction amplification. J Clin Endocrinol Metab *74*, 879-83.

Reis, S. E., Gloth, S. T., Blumenthal, R. S., Resar, J. R., Zacur, H. A., Gerstenblith, G., and Brinker, J. A. (1994). Ethinyl estradiol acutely attenuates abnormal coronary vasomotor responses to acetylcholine in postmenopausal women. Circulation *89*, 52-60.

Saadane, N., Alpert, L., and Chalifour, L. E. (2000). Altered molecular response to adrenoreceptor-induced cardiac hypertrophy in Egr-1-deficient mice. Am J Physiol Heart Circ Physiol *278*, H796-805.

Saadane, N., Alpert, L., and Chalifour, L. E. (1999). Expression of immediate early genes, GATA-4, and Nkx-2.5 in adrenergic- induced cardiac hypertrophy and during regression in adult mice. Br J Pharmacol *127*, 1165-76.

Schaible, T. F., Malhotra, A., Ciambrone, G., and Scheuer, J. (1984). The effects of gonadectomy on left ventricular function and cardiac contractile proteins in male and female rats. Circ Res *54*, 38-49.

Scheuer, J., Malhotra, A., Schaible, T. F., and Capasso, J. (1987). Effects of gonadectomy and hormonal replacement on rat hearts. Circ Res *61*, 12-9.

Schlegel, A., Wang, C., Katzenellenbogen, B. S., Pestell, R. G., and Lisanti, M. P. (1999). Caveolin-1 potentiates estrogen receptor alpha (ERalpha) signaling. caveolin-1 drives ligand-independent nuclear translocation and activation of ERalpha. J Biol Chem *274*, 33551-6.

Schwartz, K., Carrier, L., Lompre, A. M., Mercadier, J. J., and Boheler, K. R. (1992). Contractile proteins and sarcoplasmic reticulum calcium-ATPase gene expression in the hypertrophied and failing heart. Basic Res Cardiol *87*, 285-90.

Shahar, E., Folsom, A. R., Salomaa, V. V., Stinson, V. L., McGovern, P. G., Shimakawa, T., Chambless, L. E., and Wu, K. K. (1996). Relation of hormone-replacement therapy to measures of plasma fibrinolytic activity. Atherosclerosis Risk in Communities (ARIC) Study Investigators. Circulation *93*, 1970-5.

Shamim, A. (1998). Role of the female hormone estrogen in the myocardium. In Department of Biology and Department of Medicine (Würzburg: University of Würzburg).

Shamim, A., Pelzer, T., Grohe, C., and Neyses, L. (1999). Induction of Egr-1 mRNA and protein by endothelin 1, angiotensin II and norepinephrine in neonatal cardiac myocytes. Mol Cell Biochem *195*, 11-7.

Slater, A. J., Gude, N., Clarke, I. J., and Walters, W. A. (1986). Haemodynamic changes and left ventricular performance during high-dose oestrogen administration to male transsexuals. Br J Obstet Gynaecol *93*, 532-8.

Splawski, I., Tristani-Firouzi, M., Lehmann, M. H., Sanguinetti, M. C., and Keating, M. T. (1997). Mutations in the hminK gene cause long QT syndrome and suppress IKs function. Nat Genet *17*, 338-40.

Stampfer, M. J., Colditz, G. A., Willett, W. C., Manson, J. E., Rosner, B., Speizer, F. E., and Hennekens, C. H. (1991). Postmenopausal estrogen therapy and cardiovascular disease. Ten-year follow-up from the nurses' health study. N Engl J Med *325*, 756-62.

Stefano, G. B., Cadet, P., Breton, C., Goumon, Y., Prevot, V., Dessaint, J. P., Beauvillain, J. C., Roumier, A. S., Welters, I., and Salzet, M. (2000). Estradiol-stimulated nitric oxide release in human granulocytes is dependent on intracellular calcium transients: evidence of a cell surface estrogen receptor. Blood *95*, 3951-8.

Stephens, N. L., and Swynghedauw, B. (1990). Cardiovascular adaptations to mechanical overload. Mol Cell Biochem *93*, 1-6.

Sudhir, K., Ko, E., Zellner, C., Wong, H. E., Hutchison, S. J., Chou, T. M., and Chatterjee, K. (1997). Physiological concentrations of estradiol attenuate endothelin 1- induced coronary vasoconstriction in vivo. Circulation *96*, 3626-32.

Tamura, T., Said, S., and Gerdes, A. M. (1999). Gender-related differences in myocyte remodeling in progression to heart failure. Hypertension *33*, 676-80.

Watters, J. J., Campbell, J. S., Cunningham, M. J., Krebs, E. G., and Dorsa, D. M. (1997). Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen

activated protein kinase signalling cascade and c- fos immediate early gene transcription. Endocrinology *138*, 4030-3.

Weinberg, E. O., Thienelt, C. D., Katz, S. E., Bartunek, J., Tajima, M., Rohrbach, S., Douglas, P. S., and Lorell, B. H. (1999). Gender differences in molecular remodeling in pressure overload hypertrophy. J Am Coll Cardiol *34*, 264-73.

Weiner, C. P., Lizasoain, I., Baylis, S. A., Knowles, R. G., Charles, I. G., and Moncada, S. (1994). Induction of calcium-dependent nitric oxide synthases by sex hormones. Proc Natl Acad Sci U S A *91*, 5212-6.

Weiss, A., and Leinwand, L. A. (1996). The mammalian myosin heavy chain gene family. Annu Rev Cell Dev Biol *12*, 417-39.

Weisz, A., Cicatiello, L., Persico, E., Scalona, M., and Bresciani, F. (1990). Estrogen stimulates transcription of c-jun protooncogene. Mol Endocrinol *4*, 1041-50.

White, C. R., Shelton, J., Chen, S. J., Darley-Usmar, V., Allen, L., Nabors, C., Sanders, P. W., Chen, Y. F., and Oparil, S. (1997). Estrogen restores endothelial cell function in an experimental model of vascular injury. Circulation *96*, 1624-30.

Wiesner, R. J., Ehmke, H., Faulhaber, J., Zak, R., and Ruegg, J. C. (1997). Dissociation of left ventricular hypertrophy, beta-myosin heavy chain gene expression, and myosin isoform switch in rats after ascending aortic stenosis. Circulation *95*, 1253-9.

Yu, W., Dahl, G., and Werner, R. (1994). The connexin43 gene is responsive to oestrogen. Proc R Soc Lond B Biol Sci *255*, 125-32.

Zhang, Z., Yang, X. Y., and Cohen, D. M. (1998). Hypotonicity activates transcription through ERK-dependent and - independent pathways in renal cells. Am J Physiol *275*, C1104-12.