Modulation of estrogen signaling in hepatic and vascular tissue

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1. Introduction

Atherosclerosis, a pathological process characterized by vascular remodeling, is a leading cause of mortality and morbidity in the western world. Interestingly, atherosclerosis occurs rarely in premenopausal women, but rises sharply after the menopausal transition, when ovarian secretion of sex hormones is low [1-3]. This is associated with an increase in risk factors for atherosclerosis, including dyslipidaemia, insulin resistance, central obesity and hypertension in the postmenopausal period. These observations suggest that female sex steroid hormones provide protection against atherosclerosis in premenopausal women. Indeed, numerous studies have shown an atheroprotective role for estrogens. Estrogens can exert beneficial effects directly on the vessel wall, but they have also been shown to induce favorable effects on serum lipid, glucose and insulin levels [4-6]. Unfortunately, estrogens have also been postulated to induce adverse effects like endometrial cancer, breast cancer, and gallstones [7,8]. In addition, results of the Women's Health Initiative (WHI) trial regarding the vascular effects of hormone replacement therapy (HRT) have shown no demonstrable benefit of HRT [9]. Although some have criticized the design of the WHI study [10], it is also clear that an improved understanding of estrogen action in specific target tissues is required.

This thesis centers on the mechanisms of estrogen action and the effects on the development of atherosclerosis. We have focused on the liver as central organ in lipid and glucose metabolism and the vessel wall as the actual site where the injury occurs. To gain insight in tissue-specific actions of estrogens, we have spent considerable effort to develop tools for liver and blood vessel specific modulation of the estrogen receptor (ER) signaling cascade. The generation, characterization and application of these tools in vitro and in vivo will be described in the different chapters of this thesis.

2. Estrogen action

2.1 Estrogen production

17-β-Estradiol (E_2) is a steroid hormone that is primarily synthesized in the ovary of (premenopausal) women. These hormones function as an endocrine signal by exerting selective effects on distal target tissues. In addition to the female reproductive system, non-reproductive tissues such as the cardiovascular system, the immune system, the central nervous system, bone and brain are target tissues. Thus,

E_2 elicits multiple tissue-specific responses throughout the body, resulting in beneficial but also detrimental responses. In postmenopausal women, systemic E_2 production is ceased and E_2 is no longer able to function as an endocrine factor affecting distal tissues. Nevertheless, both in postmenopausal women and in men, E_2 plays an important physiological role in a number of extragonadal tissues. These tissues, which include adipose tissue, bone, numerous sites in the brain, vascular endothelial and aortic smooth muscle cells, have the capacity to express aromatase. Aromatase cytochrome P450, which is encoded by the *CYP19* gene, catalyzes the biosynthesis of E_2 and thus these tissues are able to produce E_2 themselves. However, E_2 generated via aromatase, acts predominantly at the local tissue level as a paracrine or even intracrine factor in stead of an endocrine factor [11,12]. In addition, in contrast to the ovary, these extragonadal tissues do not contain a full complement of steroidogenic enzymes [13] and therefore are dependent on substrate for aromatase activity on circulating C_{19} androgenic precursors. Because the levels of circulating androgenic precursors are lower in postmenopausal women as compared to the circulating androgenic precursors in men [14], E_2 action is lower in postmenopausal women and thus could accelerate the postmenopausal gender differences.

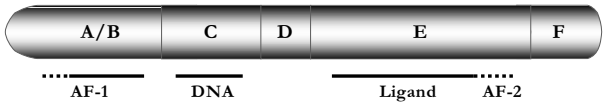


Figure 1. Functional domains of the estrogen receptor

2.2 The Estrogen Receptor

Part of the biological effects of E_2 is mediated through ERs. ERs are members of the steroid/thyroid hormone nuclear receptor superfamily that function as ligand-activated transcription factors [15]. These receptor proteins share a common architecture of six distinct domains designated alphabetically, A-F (Fig. 1) These domains are responsible for ligand binding, DNA binding and transcriptional activation [16-18]. In more detail, the amino terminus (A and B domains) contains a transcriptional activation function (AF-1) that does not require ligand for activity. In stead, it is constitutively active when linked to a suitable DNA-binding domain (DBD) [19]. This linked DBD (C domain) consists of two zinc fingers that recognize specific DNA

sequences, referred as estrogen response elements (EREs) [20]. Next to the DBD, there is a flexible hinge region (D domain) and a ligand binding domain (LBD) (E domain). The ligand binding cavity in association with the carboxy terminal region, which contains a ligand-dependent transcriptional activation domain (AF-2) (F-domain) contributes to transcription activity. Upon ligand binding, conformational changes are induced leading to an interaction surface for cofactors such as steroid receptor coactivator-1 [21]. Maximal activation of ER requires an interaction between the two activation domains AF-1 and AF-2, occurring when ligand and coactivator proteins are present [22].

The different ER domains coordinately regulate ER mediated transcription. In the initially described models, ERs reside in the cytoplasm in complex with heat shock protein 90 (HSP90). Upon ligand binding, ERs dissociate from HSP90, form dimers and interact with EREs within the promoter of their target genes to initiate transcription [23]. However, it is now clear that E_2 action is much more complex than previously thought. ERs not solely function as transcription factors, but also serve as co-activators for other transcription factors. In addition, it seems likely that they have a function outside the nucleus to mediate very rapid cellular responses to E_2. As a consequence, E_2 effects not only depend on the presence of its receptor, but also on the presence and abundance of several interactive proteins that are involved in these different ER pathways. Understanding of these multiple and cross-talking pathways (Fig. 2) in the different E_2 responsive tissues is required for mechanistic insight in the time and tissue-specific effects of E_2.

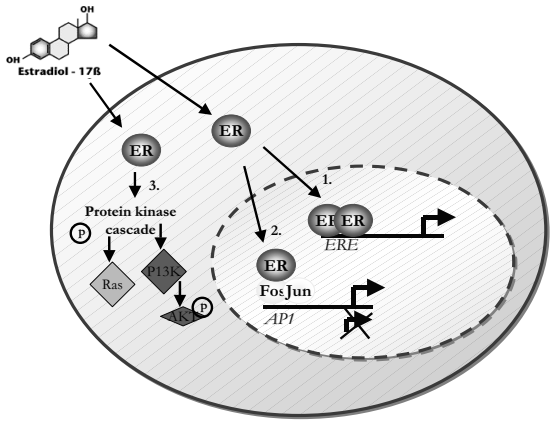


Figure 2. Estrogen receptor (ER) mediated action.
ERs are involved in several independent pathways. **1.** The classical pathway. ER initiates transcription via binding to ERE sites. **2.** The non-classical pathway. ER initiates or represses genes via interfering with other transcription factors. **3.** The non-genomic pathway. ER rapidly induces effects by activating cytoplasmic proteins (phosphorylation)

2.3 Classical ER mediated transcription

The most well-studied pathway of ER action is as ligand activated nuclear transcription factor at classical ERE sites. In this so-called classical mode of ER action, E_2 binding to cytoplasmic ER hormone induces conformational changes in the receptor, which causes dissociation of heat shock proteins that normally maintain the ER in an inactive but activatable configuration. The activated ERs are translocated to the nucleus, homodimerize and bind as dimers to two ERE half-sites that are found within the regulatory regions of their target genes. The conformational changes induced within the LBD allow the recruitment and interaction with basal transcription factors and co-activator proteins, which co-coordinately induce transcription. The ERE binding site has been discovered as a 13-base pair inverted repeat sequence (GGTCAnnnTGACC). However most of the estrogen responsive genes contain non-consensus elements, which exist as single or multiple full or half sites or they contain composite sites, consisting of EREs flanked by response elements for other transcription factors.

2.4 Non-classical ER mediated transcription

It has become apparent that ERs can also mediate transcription via a mechanism that deviates from the classical mode of action. Around one third of the genes in humans that are regulated by ERs do not contain ERE-like sequences [24]. These genes do contain alternative response elements, like AP-1 [25,26], CRE-like elements [27] and USF sites [28], from which ER can also regulate transcription. In this so-called non-classical genomic pathway, ERs do not bind directly to DNA, but modulate the function of other transcription factors through protein-protein interactions with these transcription factors or their co-activators [29]. In this complex, ER functions as a co-activator that stabilizes the DNA binding of the transcription factor complex and/or that recruits other co-activators. Several genes are known to be regulated by E_2 through this non-classical mode of ER action, including, collagenase [30], insulin like growth factor receptor 1 [31] and cyclin D1[32,33].

2.5 Non-genomic ER mediated pathway

Recently, in addition to the well-known genomic effects, E_2 mediated non-transcriptional mechanism of signal transduction have been identified. In these so-

called non-genomic pathways, the effects are very rapid, arising within seconds to few minutes from the challenge with E_2 and frequently involves activation of cytoplasmic or cell membrane bound protein kinases. The E_2 mediated non-genomic actions that have been reported include the mobilization of intracellular calcium [34], the regulation of cell membrane-ion channels [35] and of G-protein-coupled receptors [36] and activation of tyrosine kinases and mitogen activated (MAP) kinases [37]. Evidence that a distinct subpopulation of cell membrane bound ERs exist was already provided in 1977s by Pietras and Szego [38]. However, since the 90's reports have appeared that documented that ERs which were localized at the plasma membrane [39-41] could indeed exert important E_2 mediated cellular effects [42]. With respect to ligand affinity, receptor protein size, and immunological epitopes, the membrane and nuclear ERs are identical. However, since ERs do not have an intrinsic trans-membrane domain [43], the mechanism underlying membrane localization remained unidentified. Recently, it has been discovered in endothelial cells that a subpopulation of ERs is localized to the membrane via interaction with membrane-associated caveolae. Here, E_2 rapidly induces nitric oxide release via a phosphatidylinositol 3-kinase/Akt/endothelial nitric-oxide synthase (eNOS) pathway [44,45]. It has been demonstrated that palmitoylation of ER is required for this ER:protein interaction with caveolin-1 and subsequently for the receptor localization to and maintenance at the plasma membrane.

2.6 Structure of ERα and ERβ

For a long time, studies to unravel E_2 action have focused only on a single ER (nowadays referred as ERα), which was cloned and reported in 1986 [46,47]. However in 1996 a second ER, ERβ, was found [48-51].

Despite the high homology between ERα and ERβ, there is accumulating evidence that the two receptors function differently leading to distinct biological activities. These differences include, for instance, lower transcriptional activity of E_2-bound ERβ on ERE containing promoters [52,53], higher binding affinity of ERβ for the phytoestrogens coumestrol and genistein [54] and opposite actions on gene transcription, as has been observed in response to E_2 and raloxifene at AP-1 sites [55]. Molecular mechanisms for such transcriptional differences are poorly understood, but studies characterizing the structure and function relationships between the ER

subtypes have provided a molecular basis for at least some of their differential transcriptional activities. The DBD and to a lesser extent the LBD of ERα and ERβ exhibit a high degree of homology (96% and 58% amino acid identity, respectively) [56]. Likewise, functions associated with these structural domains such as ERE binding, dimerization, but also affinity to the natural estrogen E_2 are very similar for ERα and ERβ [57-60]. However, as a consequence of reduced homology in the LBD, ligands exhibiting different affinities for ERα and ERβ have also been reported [61,62]. These ligands induce ER subtype specific changes [63,64] resulting in recruitment of diverse co-activators and co-repressors. For example, affinity of ERα for SRC-3 is much higher than that observed for ERβ [65]. Thus the LBD is at least partly involved in mediating ER subtype specific actions. The amino-terminal domain, exhibiting the AF-1 region, is poorly conserved between ERα and ERβ and thus may play a significant additional role in mediating their different transcriptional activation properties. Indeed several studies provided evidence for an important role of the AF-1 region. For instance, amino-terminal deletion of the AF-1 region in ERα led to a loss of transcriptional activity induced via the classical mode of action, whereas amino-terminal deletion in ERβ resulted in an increased transcriptional activity [66]. Thus, ERα and ERβ have different transcriptional activation properties that could result at least in part from structurally divergent LBD and amino-terminal domains.

2.7 Tissue expression pattern ERα and ERβ

Since ERα and ERβ have distinct transcriptional abilities, which could even be opposite to each other, their tissue specific expression pattern is a determinant of the E_2 mediated effects. Both ERs are widely distributed throughout the body. ERα is expressed primarily in the uterus, liver, kidney, and heart, whereas ERβ is expressed primarily in the ovary, prostate, lung, gastrointestinal tract, bladder and central nervous systems. Tissues, which express both ERα and ERβ, are the mammary gland, the adrenals, bone, adipose tissue, vascular endothelium and smooth muscle cells and regions of the brain. In these tissues, there is a potential interplay between the two ERs, and thus their balance is important. For certain genes it has been found that ERβ exhibits an inhibitory activity on ERα-mediated gene expression [67-69]. It remains

to be seen whether this ERβ-dependent antagonism of ERα responses is restricted to a limited number of genes or that it represent a general mechanism in ER signaling.

3. Modulation of estrogen action

3.1 Mouse models

Mice are used as experimental models, because they are small, relatively easy to handle, have a short generation time, and, the strains are genetically defined, which reduces genetic noise. In addition, animal studies allow direct access to tissues for histological and molecular analyses. Thus, although results from mice models cannot always be extrapolated directly to humans, they provide unique mechanistic insight in the actions of E_2 and the role of the ERs.

To explore E_2 signaling, surgical and/or pharmacological manipulations, like ovariectomy (ovx) and systemic administration of estrogenic compounds have been done. Additional insight into the underlying molecular pathway of E_2 action has been obtained from ER knockout and transgenic mouse models. These models include ERα knockout ($ER\alpha^{-/-}$), $ER\beta^{-/-}$ and $ER\alpha/\beta^{-/-}$ double knockout mice [70-73] and aromatase deficient mice (ArKO) [74,75]. Of the $ER\alpha^{-/-}$ mice, two separate lines have been generated, which displayed remarkably different phenotypes. The first generated $ER\alpha^{-/-}$ mice line carries a Neo cassette in exon 1, hereafter designated as $ER\alpha_{neo}^{-/-}$ mice [76]. In these mice, the reproductive function is abolished, but several other effects of estrogen, such as estrogen induced uterine hypertrophy, persist. The persistency of these estrogenic effects is caused by the presence of a chimeric ERα protein of 55 kDa (ERα55). This chimeric ERα is able to exert transcriptional activity, although reduced when compared with the WT full-length ERα66 [77-79]. Thus, precaution has to be taken with interpretation of the data obtained using this mouse model. The second mouse line deficient in ERα was generated in 2000 by deletion of exon 2 [80], designated as $ER\alpha_{\Delta 2}^{-/-}$. These mice displayed a complete and unambiguous inactivation of ERα. Some caution has to be taken with the interpretation of data from this mouse model too, since $ER\alpha_{\Delta 2}^{-/-}$ female mice have approximately 10-fold higher levels of estrogen and also increased testosterone levels as compared to their wt counterparts [81]. In addition, a $ER\beta^{-/-}$ mouse line has been generated [82]. Those appear to have a quite normal phenotype, in which ERβ

deficiency did not affect circulating estrogen and testosterone levels. And although litter size is slightly reduced, they are able to reproduce [83].

Estrogen deficient mice have been generated by disruption of the Cyp19 gene (ArKo mice). Since they lack a functional aromatase enzyme [84], plasma E_2 levels are undetectable. Interestingly, both male and female ArKO mice have elevated plasma levels of testosterone and the luteinizing hormone, which should be taken into account when interpreting data obtained with this model.

Overall, the knockout mouse models have proven to be useful, providing valuable information about E_2 action and the nuclear receptors involved. However, insight in cell and tissue specific actions of E2 in relation to vascular disease is relatively sparse.

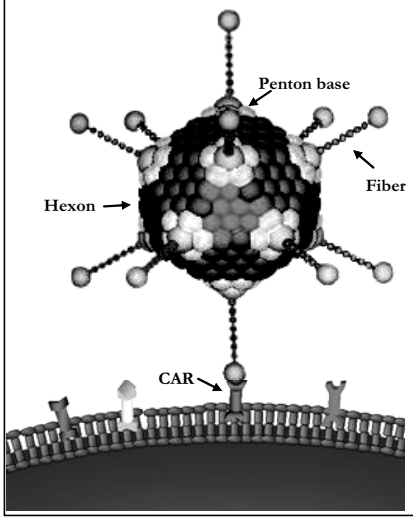
3.2 Gene transfer into liver and the vascular system

An effective strategy to modulate gene expression is by means of adenovirus (Ad) mediated gene transfer. Both wild type and constitutive active or dominant negative variants of the estrogen receptor can be delivered using Ad vectors. In general, the liver is the easiest target to accomplish gene transfer in vivo. The main reason for efficient hepatic gene transfer is the presence of a fenestrated endothelium of 100 nm width that covers the hepatic sinusoids. Consequently, macromolecules such as viral particles that are injected in the blood circulation can cross the endothelium and reach hepatocytes effortless. In addition, hepatic blood flow represents one-fifth of the cardiac output. In contrast, systemic application of vectors to deliver full-length or mutated ERs to vascular tissue is more difficult. On the one hand, treatment efficacy is decreased because vectors are sequestered by liver. On the other hand, the endothelium is refractory to transduction and forms a tight non fenestrated barrier for the underlying vascular smooth muscle cell (VSMC) layer. Thus, introduction of genes to vascular cells in vivo remains a major challenge for current gene therapy strategies.

3.2.1 Adenoviral vectors

Ad vectors are a highly efficient tool for hepatic gene transfer [85,86] and are a commonly used vector for gene delivery to the vascular system. These vectors are generated from human adenovirus serotype 5, which are non-enveloped icosahedral DNA viruses of about 90-nm diameter that can cause infections of the respiratory tracts in humans. The particle is composed of an outer capsid that contains three major components, the hexon, penton base and fiber (Fig 3). The protruding fibers consist of a knob that has a high affinity towards the coxsackie adenovirus receptor (CAR) and thus docks the particle to CAR expressing host cells [87-89]. After this initial binding, the RGD motifs on the penton base interact with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins, which leads to clathrin-mediated endocytosis of the virus particle [90-92]. Once endocytosed, the virus escapes the endosome to enter the nucleus. Once the virus has passed its genome to the nucleus, selective transcription and translation are initiated. First, the virus modulates the function of the host cell to facilitate its replication, transcription and translation of the viral genome. Then, the newly synthesized viral components are assembled into new viral particles, which will be released upon cell lysis. These can then initiate a new round of infection and viral replication.

Figure 3. Adenovirus Morphology



To use Ad5 as a delivery device, recombinant Ad vectors have been rendered replication-deficient and less immunogenic by removing the E1 and E3 regions. These regions are essential for the activation of replication of the viral genome and the initiation of a host immune response, respectively. The essential E1 functions are complemented in trans by means of specific cell lines that constitutively express the E1 proteins, such as the 293, 911 and PerC6 cell lines [93,94]. Subsequently, up to 6.5 kb of foreign coding DNA can be introduced into the E1/E3 deleted vector. To transfer the transgene to a particular cell type, the expression pattern of CAR and A5B3 integrins are essential. Although many cell types can be infected with

adenovirus vectors in vitro, for refractory cell types this requires high multiplicities of infection (MOI). High MOI's are associated with cytotoxicity that may interfere with the interpretation of the results.

3.2.2 Targeting adenoviral vectors

Vascular cells express very little, if any CAR and are thus refractory to Ad mediated infection. To improve gene delivery to vascular cells in terms of efficiency (achieve gene transfer to a high percentage of cells with low doses and low immunogenicity) and selectivity (diminish affinity for non-target sites), Ad vectors have been engineered. Two different approaches are used to target transgene expression to alternative non-CAR expressing cells such as endothelial cells (EC) and VSMCs. The first approach modifies the viral capsid through genetic alteration, for example by engineering endothelium-binding peptides into the Ad fiber protein [95,96], or by psuedotyping (exchange of Ad fiber for a fiber from an alternative serotype possessing a more favourable cell binding profile) [97]. The second approach employs bi-valent molecules where one part of the molecule binds to the vector and the other part of the molecule will target the complex to an alternative receptor that is expressed at the surface of the desired target tissue. A commonly used example of a bi-valent molecule is the bispecific antibody [98,99]. In addition to targeting, tissue specific expression can be enhanced by using promoter/enhancer sequences from endothelium- or VSMC-restricted genes [100]. The endothelial specificity of minimal promoters derived from Tie II (angiopoietin receptor), von Willebrand factor, fms-like tyrosine kinase-1, thrombomodulin, E-selectin and ICAM-2 have been demonstrated by transgenic mouse models expressing lacZ driven by these promoters.

4. Estrogen action in the vascular system

4.1 The vessel wall

The vessel wall consists of three well-defined layers: the innermost layer is called the endothelium, the middle layer is called the media, and the outermost layer is known as the adventitia (Fig 4A). Of these three layers, the endothelium is separated from the media by the internal elastic lamina and the media is separated from the adventitia by the external elastic lamina. The endothelium consists of a single contiguous lining of endothelial cells that forms the barrier between the blood

flow and the artery. It has become evident that this endothelium is not a passive barrier. On the contrary, it plays a major role in several processes, including maintaining vascular homeostasis, controlling vascular permeability, inhibiting platelet adhesion and aggregation and limiting activation of the coagulation system. The media consists of VSMC and an extracellular matrix (ECM). The major role of VSMC is to regulate blood pressure and thus blood flow. The outermost layer of the artery, the adventitia, consists of loose matrix of elastin, smooth muscle cells, fibroblasts and collagen.

4.2 Role of estrogen in vascular tone

Vascular tone and function seem to differ between men and women, as women have lower blood pressure than age-matched males [101]. Moreover, hypertension occurs with higher frequency in men and postmenopausal women than in premenopausal women. In part this has been related to the presence of endogenous estrogens, as healthy men treated with aromatase inhibitor displayed impaired vascular dilatation [102,103]. Vascular tone is regulated by a complex set of vasodilator and vasoconstrictor factors that adjust the contractile state of VSMC [104,105]. The endothelium is mainly responsible for the synthesis and secretion of these factors, including angiotensin II, endothelin-1 and NO. In humans, the endothelium-dependent vasodilatory effect of E_2 could at least be partly explained by its enhancement of NO production [106]. Moreover, in vitro studies have confirmed that the endothelial mediated NO release is increased by E_2. This release occurred through both the ERα mediated classical genomic pathway as well as through the rapid non-genomic pathways [107-109]. Recent data have demonstrated that in addition to ERα, ERβ is involved in the regulation of endothelial NO production. Both the ERβ- as well as the ERα-selective agonist, DPN and PPT rapidly induced eNOS activity in EC [110].

The contractile response of the underlying VSMC layer can also be modulated in an endothelium-independent manner. By denudation (stripping of the endothelial layer) of the vessel wall, it has been shown that E_2 is capable of reducing vasoconstriction in an endothelium-independent manner [111]. A predominant role for the E_2 mediated vascular dilatation in endothelial-denuded vessels seemed to be played by ERβ. In mice, ERβ deficiency led to a nearly two-fold enhancement of

phenylephrine -induced vasoconstriction compared to wt controls. In addition, blood pressure was increased in ERβ$^{-/-}$ mice. Inducible NOS (iNOS) appears to be involved in ERβ mediated vascular dilatation. In E_2 treated denudated vessels, enhanced expression of iNOS protein was detected [112,113], whereas reduced iNOS protein levels was observed in aorta of ERα$_{neo}^{-/-}$/ERβ$^{-/-}$ mice [114]. The effect of ERβ on iNOS expression seems to be induced by VSMCs, as demonstrated by an in vitro iNOS promoter study [115]. Overall, E_2 induced stimulation of endothelium dependent and independent vascular relaxation may contribute to the observed gender differences in vascular tone. Depending on the vascular cell type, ERα and ERβ seem to have opposite effects and/or could exert subtype specific effects.

4.3 Role of estrogen in vascular injury

An intact vascular endothelium is critical to the maintenance of normal arterial tone and provides an anti-inflammatory, anti-coagulatory surface. In the case of injury of the endothelium, caused by a wide range of genetic and environmental factors like elevated levels of LDL cholesterol, obesity, diabetes mellitus, cigarette smoking, and exposure to infectious agents [116], EC-activation and VSMCs proliferation are initiated (Fig 4B). These processes are thought to be the precursor of vascular pathologies, including atherosclerosis and restenosis [117,118].

In mouse models, vascular injury can be obtained by denudation of the carotid artery. In this model E_2 has been demonstrated to inhibit VSMC proliferation [119,120]. To clarify the role of ERs in the protective mechanism of E_2 after vascular injury, both ERα$^{-/-}$ and ERβ$^{-/-}$ mice have been used. In wt as well as in ERα$_{neo}^{-/-}$ and ERβ$^{-/-}$ mice, E_2 still attenuates injury induced VSMC proliferation [121,122]. In contrast, in the follow-up study were ERα$_{\Delta 2}^{-/-}$ mice have been used, E_2 was no longer protective [123]. Thus, ERα is involved in E_2 mediated inhibition of VSMC proliferation after vascular injury and the chimeric ERα present in the aorta of ERα$_{neo}^{-/-}$ mice [124] is sufficient to confer complete protection by E_2. Remarkably, in the absence of E_2, ERα$_{\Delta 2}^{-/-}$ mice displayed significantly smaller vascular injury responses as compared to wt and ERβ$^{-/-}$ mice [107, 108]. This signifies either a potential harmful role for E_2-independent ERα mediated activity in the vascular injury response, or in the absence of E_2, ERβ has a beneficial role, which in wt mice is overshadowed by ERα. Rapid restoration of endothelial integrity and reduction of

endothelial activation has a favorable impact on VSMC proliferation [125,126] and thus potentially could reduce the vascular injury response. The E_2 induced attenuation of the response to injury might be due to enhanced re-endothelialization of the damaged arterial segment. Indeed, by use of wt, $ER\beta^{-/-}$ and $ER\alpha_{\Delta 2}{}^{-/-}$ mice models it has been demonstrated that E_2 accelerates endothelial re-growth via ERα [127]. In general, the ability of the endothelium to renew depends on the migration of surrounding mature EC, but also on the attraction and adhesion of circulating endothelial progenitor cells (EPCs) to the injured region, which then differentiate into endothelial-like cells. E_2 has been shown to increase the number of EPCs in the

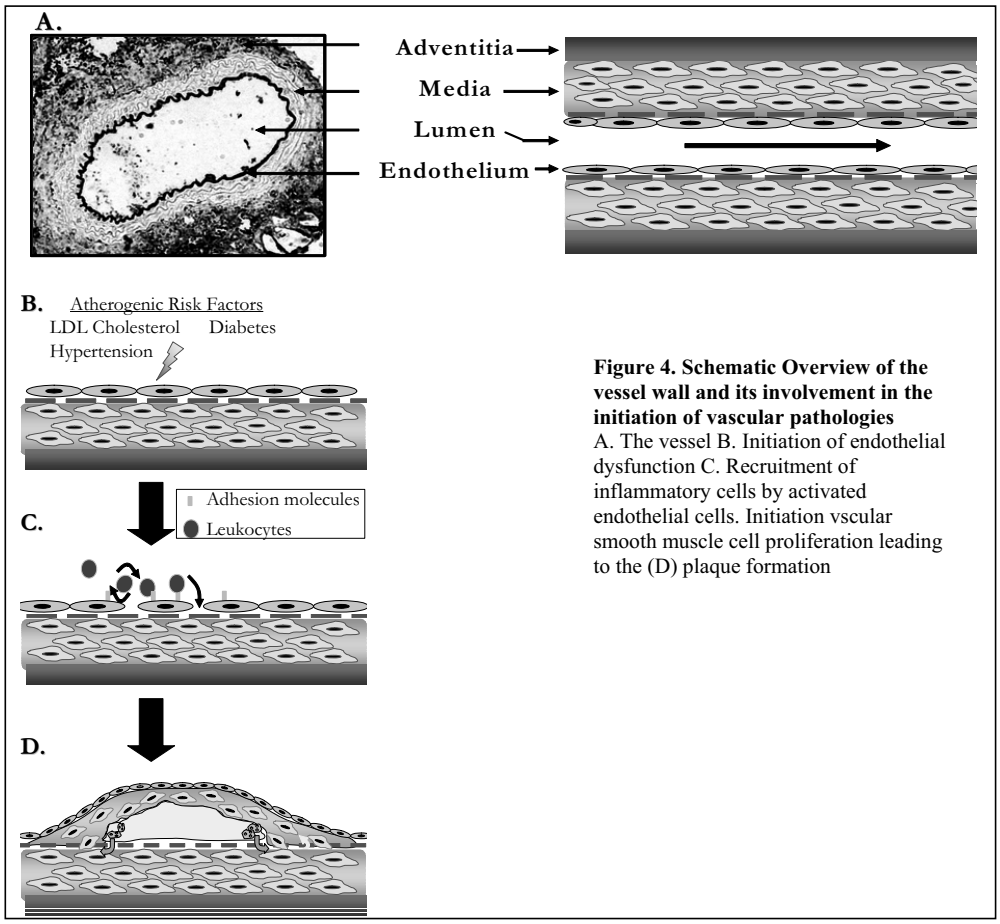


Figure 4. Schematic Overview of the vessel wall and its involvement in the initiation of vascular pathologies
A. The vessel B. Initiation of endothelial dysfunction C. Recruitment of inflammatory cells by activated endothelial cells. Initiation vscular smooth muscle cell proliferation leading to the (D) plaque formation

circulation but also at the site of vascular lesion. As a consequence, the vascular injury response has been reduced [128]. Thus, the protective vascular effects of E_2 are at least partly due to effects on circulating EPCs. Accordingly, the available mouse models of estrogen deficiency provide evidence that E_2 mediated activation of ERα reduces the vascular injury response. However, whether this effect fully accounts on the enhanced attraction and adhesion of circulating EPCs or whether there is also an effect locally at the surrounding mature ECs remains to be addressed.

4.3.1 Atherosclerosis

Vascular injury is an important initial step in the development of atherosclerosis, a progressive disease in which fat and cholesterol are deposited along artery walls (Fig. 4C). In short, due to vascular injury, permeability and expression of endothelial adhesion molecules is enhanced. Consequently, circulating monocytes and lymphocytes interact with the vessel wall. These inflammatory cells secrete cytokines and chemokines (chemoattractive cytokines), which initiate a whole spectrum of reactions leading to vascular smooth muscle cell hyperplasia, intimal migration and further accumulation of lipids. If the damaging insult persists, the inflammatory process may become chronic, the fibro proliferative response persists and lipids continue to accumulate within the vessel wall. Eventually, the enlarged fatty lesion may restrict blood flow through the blood vessel, increasing the risk of heart attack and stroke.

To study the role of E_2 in the pathogenesis of atherosclerosis, atherosclerosis-prone mouse models, including apolipoprotein E (ApoE) knockout and low-density lipoprotein (LDL) receptor (Ldlr) knockout mice have been used. In ovariectomized (ovx) $ApoE^{-/-}$ female mice, systemic administration of E_2 resulted in a consistent and dramatic inhibition of lesion initiation and progression [129-131]. In addition to its inhibitory effect in females, estrogen appears to be equally efficacious in males. For example, Nathan and coworkers [132] have shown that orchidectomy increased lesion size in $Ldlr^{-/-}$ males, which was reversed by exogenous administration of either E_2 or testosterone. Co-administration of an aromatase inhibitor, on the other hand, removed the atheroprotective effect of exogenous testosterone, suggesting that local conversion of testosterone to E_2 in vascular cells attenuates atherosclerosis in male mice. In addition, in the aorta of streptozotocin-induced hyperglycemic $Apoe^{-/-}$ males, E_2

reduced lesion size and prevented calcified cartilaginous metaplasia [133]. The observed E_2 mediated inhibition of lesion size was in some studies associated with a reduction in total plasma cholesterol levels, [134-136], but not in all [137,138]. Thus, E_2 possesses cardiovascular protective actions beyond an effect on plasma lipids, most likely via direct effects on the vessel wall.

The atheroprotective action of E_2 could be established trough ERα and ERβ, as both ERs are present in VSMC and EC. Absolute expression levels of ERs in diverse vascular beds and between the two sexes have not been characterized yet. But, the overall expression level in vascular cells is low. Moreover, ERs are absent from various vascular cells kept in culture, which complicates the analsysis of the role of ERs in the vasculature. Thus far, to investigate the relative contribution of each receptor in the atheroprotective role of E_2, $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice crossbred with $ApoE^{-/-}$ mice have been used. The inhibitory effect of E_2 on atherosclerotic lesion progression obtained in $ApoE^{-/-}$ females was almost completely abrogated in $ER\alpha_{neo}{}^{-/-}$ $Apoe^{-/-}$ mice [139]. In addition, the plasma lipid-lowering effect of E_2 was eliminated. However, fibrous caps and other advanced lesion characteristics were reduced in E_2 treated $ER\alpha_{neo}{}^{-/-}Apoe^{-/-}$ as compared to control $ER\alpha_{neo}{}^{-/-}Apoe^{-/-}$ mice [140]. Probably, this residual protective effect is mediated by the presence of the chimeric ERα protein. Conversely, it has been found that in $ER\beta^{-/-}Apoe^{-/-}$ mice, E_2 treatment inhibited atherosclerotic lesion progression equally as compared to $Apoe^{-/-}$ females. Thus E_2 is fully atheroprotective in the absence of ERβ (reviewed in [141], manuscript data in preparation), demonstrating that at least at early stages of plaque formation, the anti-atherogenic effect of E_2 is primarily mediated through ERα and independent of ERβ.

4.3.2 Restenosis

Occlusion of the artery, as occurs in atherosclerotic vessels, can be mechanically treated. The most commonly used therapy of atherosclerotic complications consists of percutaneous transluminal coronary angioplasty (PTCA) followed by endovascular stent implantation [142]. This procedure depends on a catheter containing a deflated balloon. Once the catheter is passed into the narrowed part of the artery, the balloon is inflated allowing more blood flow. The immediate results are good, but as a consequence of constrictive remodeling and formation of a

neointima rich in proliferating SMC and ECM, restenosis occurs within a few months in 30–50% of treated patients. An implanted stent, a spring-like device designed to push open the artery, can prevent constrictive remodeling. However, neointimal proliferation still occurs and is responsible for restenosis in 20–30% of the stent-treated patients. [143,144]. Currently, to prevent intrastent restenosis, stents have been coated with the anti-mitotic drug, Rapamycine or Taxol, which seems very efficient in preventing neointimal hyperplasia. However, these drugs also inhibit the re-endothelialization process, as was demonstrated in large animal models [145].

There is currently considerable attention for drugs that favor re-endothelialization, including drugs that act on the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-1 or –2. However, these drugs have failed due to pleiotropic and deleterious effects. Within this context E_2 has also been considered. The vascular injury models have already demonstrated its anti-mitotic and endothelial re-growth properties [146,147]. In addition, pig models have been used, which displayed improved endothelial function, enhanced re-endothelialization and decreased neointima formation after intra-muscular injections of E_2 during stenting [148], but also after local delivery of E_2 during percutaneous transluminal coronary angioplasty and stenting [149-151]. In humans, a pilot study with E_2-eluting stents has been performed, which did not demonstrate deleterious affects [152]. A randomized follow-up study is required to fully evaluate the potential benefit of E2-coated stents. At the moment, the underlying mechanism and the subsequent involvement of ERα and ERβ are unknown and receptor-specific ligands may have differential effects.

5. Estrogen and Lipid & Glucose Metabolism

5.1 Lipid & glucose metabolism

Hyperlipidemia and insulin resitance are major risk factors for the development of cardiovascular disease.The body has developed a sophisticated lipoprotein and glucose transport system to meet to the diverse demands from different tissues under different conditions. These two systems are heavily interconnected and excess energy intake or genetic defects can deregulate lipid and glucose metabolism, leading to hyperlipidemia and insulin resistance and increased risk for cardiovascular disease. Insulin resistance is characterized by a diminished biological effect of insulin on glucose and free fatty acid (FFA) uptake by skeletal

muscle and adipose tissue, respectively and the suppression of glucose output by the liver (via decreased glyconeogenesis and glycogenolysis).

The liver forms the central site of lipid and glucose metabolism and therefore, plays an essential role in the maintenance of whole body energy homeostasis. It removes remnant lipoproteins from, and delivers newly synthesized lipoproteins to the bloodstream. To maintain the fairly steady concentration of glucose in the blood, the liver takes up and releases glucose into the bloodstream. Furthermore, it expresses a well-orchestrated network of genes that maintain the intra-hepatic cholesterol and glucose homeostasis. It is the main organ involved in de novo FA synthesis. Newly synthesized FA can be converted into triglycerides (TG) to be stored or secreted as VLDL-TG. FA can also be used for energy production via β-oxidation. Glucose can be produced directly through gluconeogenesis from non-carbohydrate sources like amino acids, glycerol and lactate. The liver is also able to produce glucose through phosphorylation of glycogen, the storage form of glucose. This process is called glycogenolysis. On the other hand, when blood glucose levels are high, the liver will function as reservoir to take up and convert the excess of glucose into glycogen for future needs.

5.2 Effects of estrogen on lipid and glucose metabolism

Estrogens seem to be implicated in whole body energy homeostasis. Both gender and menopausal status influence lipid and glucose metabolism [153-155]. For example, menopause is associated with lipid abnormalities. Moreover, menopause is also associated with fat accumulation in the abdominal regions, which again is associated with increased plasma FFA and decreased adiponectin levels, both important components of the insulin-resistance syndrome [156,157]. The importance of estrogens has been revealed by individuals that carry mutations in the gene encoding aromatase. They develop obesity, insulin resistance, hypercholesterolemia, and hypertriglyceridemia [158-162]. Models of estrogen deficiency have been used to obtain more insight. ArKO mice age-progressively develop hypercholesterolemia, hyperleptinemia, and become obese. By 1 yr of age, ArKO males also exhibit elevated plasma triglyceride levels and develop hepatic steatosis [163]. MRI data of ArKO mice reveal that females have three times and males have twice as much adipose tissue as compared to wt mice. ER deficient models have highlighted the importance

of ERα and ERβ in lipid and glucose metabolism. Both ERα$^{-/-}$ and ERα/ERβ$^{-/-}$ mice develop a lipid phenotype similar to the ArKOs, whereas no lipid phenotype is described in ERβ$^{-/-}$ mice [164,165]. ERα deficiency also results in insulin resistance, glucose intolerance, and adipose hyperplasia and hypertrophy in both sexes, as studied in ERα$_{neo}^{-/-}$ [166]. This seems to be comparable with the human situation. One adult male with ERα deficiency has been described [167] and the clinical features of this patient include glucose intolerance, hyperinsulinemia, and lipid abnormalities [168,169]. Interestingly, a role of ERβ was indicated by estrogen depletion (ovx) and exogenous E_2 treatment of ERα$_{neo}^{-/-}$ mice. These experiments demonstrated that removing of the E_2/ERβ signaling cascade by ovx resulted in reduced body and fat-pad weights and adipose size, which could be reversed by E_2 treatment. This indicates that ERβ mediates effects on adipose tissue that are opposite to those of ERα [170]. In addition, estrogen depletion of ERα$_{neo}^{-/-}$ mice improved glucose tolerance and insulin sensitivity, suggesting a harmful role for ERβ in glucose metabolism. Thus, a clear physiological role in the regulation of lipoprotein metabolism in mice has been ascribed to ERα, whereas both ERα and ERβ influence glucose metabolism. However, it should be mentioned that ERα most likely plays the most dominant role in glucose metabolism, since thus far the role of ERβ is only apparent under ERα deficient conditions.

5.3 Role of estrogen in the liver

In the liver, estrogens can enhance liver regeneration and suppress liver fibrosis [171,172]. However, the involvement of estrogens in the hepatic lipid and glucose signaling cascade is less clear. Relatively few reports have appeared in the literature, focusing on hepatic lipid and glucose regulated genes. Of the two ERs only ERα is expressed in liver [173-175], which is in accordance with the fact that ERα seems to play a more important role in lipid metabolism than ERβ [176,177]. The involvement of estrogens and ERα in the regulation of intra-hepatic lipid levels has been demonstrated in ArKO, ERα$_{neo}^{-/-}$ and ERα$_{\Delta 2}^{-/-}$ mice. In all these models analysis of their hepatic lipid content revealed a 3- to 5-fold increase in the TG level [178,179]. In addition, 6 weeks of E_2 treatment in ArKO males effectively blocked the development of hepatic steatosis. Molecular characterization of ArKO mice revealed

that the intra-hepatic signaling pathway was disturbed towards a situation of both enhanced input (enzymes involved in fatty acid synthesis were increased) as well as reduced output (enzymes involved β-oxidation were decreased). These data demonstrate that estrogens do seem to play an important role in hepatic lipid and carbohydrate metabolism, however because the hepatic lipid phenotype in the ArKO and ER$\alpha^{-/-}$ mice is still sex dependent, it seems likely that estrogens are not the sole determinant of the gender-related differences.

A small number of studies have gained more insights in the (direct) effect of estrogens on hepatic genes regulating glucose and lipid homeostasis. The orphan short heterodimer partner (SHP) appears to be induced by chronic [180], but also instant administration of estrogen [181] in liver of wt mice. However, induction of SHP did not inhibit expression of the known SHP target genes cholesterol 7α-hydroxylase (CYP7A1) or sterol 12α- hydroxylase (CYP8B1) and thus the biological implication of estrogen induced expression of hepatic SHP remains to be determined. SR-BI and SR-BII are both HDL receptors involved in the internalization of HDL cholesterol esters, with SR-BII being approximately 4-fold less efficient than SR-BI. Rat studies have found E_2 mediated regulation of hepatic SR-BI and SRBII expression levels [182,183]. However, the underlying mechanism and its impact on HDL metabolism is unclear. Hepatic lipase (HL) participates in the uptake of HDL particles by hepatocytes. E_2 has been shown to increase HL mRNA as well as HL activity with the concomitant lowering of plasma levels of HDL [184]. Apo A-I is the major protein constituent of HDL and has been attributed to its cardioprotective effect [185,186]. Estrogens have been shown to induce Apo A-I promoter activity and gene expression [187-189]. In summary, E_2 clearly affects lipid and glucose metabolism. Although some studies have reported hepatic lipid target genes, the role of liver is not thoroughly known.

6. Thesis Outline

In this thesis we have addressed the role of estrogen signaling in liver and vessel wall with emphasis on the link with vascular disease. To study E_2 signaling in selected tissues, we set out to develop tools to modulate the E_2 signaling cascade in a tissue-specific manner. In chapters 2-4, we have focused on the liver and addressed the physiology of estrogen signaling in the development of metabolic disorders. In

chapter 2, we have generated short interfering RNA constructs to down-regulate mouse ERα mRNA levels. By producing Ad vectors expressing shRNA against mERα (Ad.shERα), we generated a model to study the role of hepatic ER signaling. Both hepatic ERα RNA levels, as well as hepatic ERα activity were monitored in time and found to be significantly decreased. The Ad.shERα is further explored in **chapter 3**, in which the effect of hepatic ERα repression on lipid metabolism has been analyzed. Ad.shERα was intravenously injected in APOE*3-Leiden mice, a mouse model for hyperlipidemia. After several days, when hepatic ERα RNA and protein levels were significantly down-regulated, hepatic VLDL-TG production, lipid levels, and mRNA levels of relevant lipid-related genes were analyzed. Surprisingly, we found that the hepatic ERα levels are not a limiting factor in lipid metabolism. In **chapter 4**, we have studied the acute effect of E_2 on insulin sensitivity. Although E_2 was applied systemically, we found by using a sophisticated in vivo imaging setup that exclusive and maximal activation of hepatic ER was achieved six hours after E_2 administration. Taken into account that the effects were examined after this short period of time, this study provides evidence for a role for hepatic ERα in maintaining glucose homeostasis.

In chapters 5-8 of this thesis, we set out to develop models to modulate estrogen signaling in the vessel wall. In **chapter 5**, Ad vectors have been targeted to enhance gene transfer to transformed as well as to primary vascular cells. The targeting approach is based on a bi-functional linker construct, which contains the extra cellular domain of the Ad receptor linked to a cRGD peptide. This resulted in a targeting construct that binds to the Ad vector at one side and to $\alpha_v\beta_{3/5}$ integrins at the other site. Both primary as well as transformed vascular cells were infected with a high efficiency using this construct. In a subsequent study, we set out to target Ad vectors to the carotid artery of mice in vivo. **Chapter 6** describes the work that has been performed to obtain vascular gene transfer in vivo. Although de-targeting of the liver was achieved successfully, targeting using two independent ligands failed to redirect tropism of the Ad vectors. Experiments indicate that stability of Ad in the circulation may be an important limitation. In **chapter 7**, the effect of E_2 on the expression of adhesion molecules in EC in presence of normal and reduced ERα levels has been analyzed. In this study, we have generated shERα expressing lentiviral vectors that result in persistent reduction of ERα levels. These data

demonstrate that E_2 reduces the expression of adhesion factors, suggesting an anti-inflammatory role for E_2. In this response, ERα is required but not a rate limiting factor. **In chapter 8,** we evaluated the specific role of ERα and ERβ in the vascular wall in vivo. A non-constrictive drug-eluting collar was placed around the femoral artery of mice, which simultaneously induces intimal proliferation and releases either placebo, ERα or ERβ specific agonists. These data demonstrated that in adition to ERα, ERβ is able to inhibit neointima formation. In the last chapter, **chapter 9**, the findings presented in this thesis and possibilities for future research are discussed.

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