



**University of
Zurich**^{UZH}

**Zurich Open Repository and
Archive**

University of Zurich
Main Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2008

Role of Peroxisome Proliferator-Activated Receptor and Estrogen Receptors in Mediating the Anti-Mitogenic Actions of Phytoestrogens

Boogen, E M

Abstract: Most Western countries face high and increasing rates of cardiovascular disease (CVD). Each year, heart disease kills more people than cancer. The increasing risk of CVD in woman after post-menopausal changes suggests that estrogen has cardioprotective effects. It is well known that estrogen binds to estrogen receptors (ER) and mediates its effects via different pathways and thereby influences various systems in the male and female physiology. Phytoestrogens, natural constituents of many plants, seeds and grains are oestrogen like ligands with numerous effects to the human body. They are similar to estrogens and bind to ERs, they are supposed to mimic the effects of estrogens and mediate these activities via ERs. However, recent findings suggest that phytoestrogens bind to PPAR- receptors. Together with the fact, that the protective effects of the phytoestrogen genistein are not blocked by ER antagonist and that PPAR- agonists are also cardioprotective we investigated, whether the protective action of phytoestrogens may be PPAR- mediated.

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-16272>

Dissertation

Published Version

Originally published at:

Boogen, E M. Role of Peroxisome Proliferator-Activated Receptor and Estrogen Receptors in Mediating the Anti-Mitogenic Actions of Phytoestrogens. 2008, Clinic for Reproductive Endocrinology, Faculty of Medicine.

Role of Peroxisome Proliferator-Activated Receptor γ and Estrogen Receptors in Mediating the Anti-Mitogenic Actions of Phytoestrogens

Abstract

Most Western countries face high and increasing rates of cardiovascular disease (CVD). Each year, heart disease kills more people than cancer. The increasing risk of CVD in woman after postmenopausal changes suggests that estrogen has cardioprotective effects. It is well known that estrogen binds to estrogen receptors (ER) and mediates its effects via different pathways and thereby influences various systems in the male and female physiology. Phytoestrogens, natural constituents of many plants, seeds and grains are oestrogen like ligands with numerous effects to the human body. They are similar to estrogens and bind to ERs, they are supposed to mimic the effects of estrogens and mediate these activities via ERs. However, recent findings suggest that phytoestrogens bind to PPAR- γ receptors. Together with the fact, that the protective effects of the phytoestrogen genistein are not blocked by ER antagonist and that PPAR- γ agonists are also cardioprotective we investigated, whether the protective action of phytoestrogens may be PPAR- γ mediated.

Universitätsspital Zürich
Departement Frauenheilkunde
Vorsitzender: Prof. Dr. med. R. Zimmermann
Klinik für Reproduktionsendokrinologie
Direktor: Prof. Dr. med. B. Imthurn

Arbeit unter der Leitung von Prof. Dr. sc. nat. R. K. Dubey

Role of Peroxisome Proliferator-Activated Receptor γ and Estrogen Receptors in Mediating the Anti-Mitogenic Actions of Phytoestrogens

INAUGURAL-DISSERTATION
zur Erlangung der Doktorwürde der Medizinischen Fakultät
der Universität Zürich

vorgelegt von
Eva-Maria Boogen
von Deutschland

Genehmigt auf Antrag von Prof. Dr. med. B. Imthurn
Zürich 2008

Contents

Abstract	3
Abbreviations	5
1. Introduction	6
1.1. Nuclear receptor group and Peroxisome Proliferator Activated Receptors	6
1.2. Phytoestrogens – chemical structure, nomenclature and sources	7
1.3. Absorption, distribution, metabolism and excretion of phytoestrogens	8
1.4. oestrogen action and receptor mechanism	11
1.5. Effects mediated by phytoestrogens	17
2. General hypothesis and objectives	21
3. Material and Methods	21
3.1. Human Aortic Smooth Muscle Cell Culture (HASMCs)	21
3.2. Cryopreservation of Cells	21
3.3. Cell Thawing	22
3.4. DNA Synthesis	22
3.5. Collagen Synthesis	23
3.6. Cell number	23
3.7. Statistical Analysis	23
4. Results	24
4.1. Effects of genistein on DNA synthesis	24
4.2. Effects of genistein on collagen synthesis	25
4.3. Effects of genistein on SMC proliferation	27
4.4. The effect of Rosiglitazone and Genistein on SMC growth via PPAR-γ	28
4.5. Antiproliferative Actions of Estradiol, Rosiglitazone and phytoestrogens	30
5. Discussion	32
5.1. Clinical background	32
5.2. Effects via PPAR-γ receptor	34
5.3. Perspectives	39
Attachments	41
References	42
Acknowledgements	49
Curriculum vitae – Eva-Maria Boogen	50

Abstract

Introduction

Most Western countries face high and increasing rates of cardiovascular disease (CVD). Each year, heart disease kills more people than cancer. The increasing risk of CVD in woman after postmenopausal changes suggests that estrogen has cardioprotective effects. It is well known that estrogen binds to estrogen receptors (ER) and mediates its effects via different pathways and thereby influences various systems in the male and female physiology.

Phytoestrogens, natural constituents of many plants, seeds and grains are oestrogen like ligands with numerous effects to the human body. They are similar to estrogens and bind to ERs, they are supposed to mimic the effects of estrogens and mediate these activities via ERs. However, recent findings suggest that phytoestrogens bind to PPAR- γ receptors. Together with the fact, that the protective effects of the phytoestrogen genistein are not blocked by ER antagonist and that PPAR- γ agonists are also cardioprotective we investigated, whether the protective action of phytoestrogens may be PPAR- γ mediated.

Material and Methods

Human aortic smooth muscle cells were harvested and cultured under standard conditions and plated in 24 well tissue culture dishes. Cultures were either treated with genistein, daidzein, equol, resveratrol and rosiglitazone. Each experiment was performed with or without the use of oestrogen receptor antagonist ICI 182,780 and PPAR- γ antagonist GW 9662.

After 20h different experiments were performed. Collagen synthesis, DNA synthesis and total cell numbers were measured from standardized numbers of SMCs from each cell culture.

SMCs with no treatment served as control.

Results

SMCs treated with genistein showed a significant inhibition of DNA synthesis, while additional PPAR- γ antagonist treatment lead to reversal of the inhibitory effect of genistein on DNA synthesis. In presence of oestrogen receptor antagonist ICI182780 the inhibitory effects of genistein remained unchanged.

Collagen synthesis was significantly reduced in SMCs treated with genistein alone, while these effects were reversed in the presence of PPAR- γ receptor antagonist. Again, the inhibitory effects of genistein on collagen synthesis were not abrogated in presence of oestrogen receptor antagonist. SMC proliferation assays showed a decreased level of proliferation rate under genistein treatment and these effects were not altered by ER antagonist. Genistein treatment inhibited SMC proliferation and these effects were blocked by PPAR- γ receptor antagonist.

Compared to rosiglitazone, genistein was able to lower SMC numbers in culture to a significant higher degree depending on the concentration applied. In comparison to other oestrogen-like structures (daidzein, equol, resveratrol), genistein had the biggest impact on lowering DNA synthesis rate in SMCs, when PPAR- γ receptor was blocked.

Discussion

The medical and economical relevance of atherosclerosis and CVD cannot be overrated. The high incidence in the western world, as well as the high mortality rate caused by heart attack underline the need of new and modern therapy concepts. After menopausal changes in the hormonal system – either by surgical intervention or by normal aging – the risk of CVD increases rapidly in femals. Hormone replacement therapy can protect the cardiovascular system from effects, associated with CVD and atherosclerosis. Until now it was believed, that oestrogen or estrogen like molecules mediate protective effects via the estrogen receptors (ER). Our studies show that certain effects of phytoestrogens on smooth muscle cells from the human aorta are not mediated via ERs but via PPAR- γ receptor. Even when ERs are completely blocked, cell proliferation parameters and collagen synthesis can be decreased following genistein treatment. Compared to different phytoestrogens, genistein has the most powerful impact and is therefore a promising target drug for hormon replacement in this term.

Abbreviations

HSP:	heat shock proteins
ER:	oestrogen receptor
ERE:	oestrogen response element
AP-1:	activating protein 1
SMC:	smooth-muscle cell
vSMC:	vascular smooth-muscle cell
PE:	phytoestrogens
CVD:	cardiovascular disease
HDL:	high-density lipoprotein
HEGF:	human epidermal-growth-factor
LDL:	low-density lipoprotein
HRT:	hormon replacement therapy
HSP:	heat shock proteins
ERE:	oestrogen responsive elements
PPAR- γ :	peroxisome proliferator-activated receptors
MAPK:	mitogen-activated-protein-kinase
SHBG:	sex-hormone-binding-globulin
TCA:	trichloroacetic acid
TZD:	thiazolidinediones

1. Introduction

1.1. Nuclear receptor group and Peroxisome Proliferator Activated Receptors

Nuclear receptors are one of the biggest groups of transcription factors, being activated through the binding of hydrophobic ligands. Within this family the group of Peroxisome Proliferator Activated Receptors (PPAR) contains three members: PPAR- α , PPAR- γ , and PPAR- δ . These receptors can be activated by both, synthetic and natural ligands such as eicosanoids, free fatty acids, lipid-lowering drugs and thiazolidinediones (TZDs).

PPAR- α is expressed in liver, heart, kidney, muscle and adipose tissue. When activated, the expression of apolipoprotein in the liver is increased, which subsequently leads to an inhibition of lipoprotein lipase (LDL). In the development of atherosclerosis, PPAR- α mediated effects in endothelial cells and smooth muscle cells (SMCs) inhibit inflammatory responses. Several factors like NO-synthase, monocyte chemotactic protein 1 (MCP-1), interleukin-6 and others are downregulated following activation of PPAR- α .

PPAR- γ , the second member of the PPAR family has three isoforms: PPAR- γ 1, PPAR- γ 2 and PPAR- γ 3. While isoforms 1 and 3 encode the same polypeptide, isoform 2 contains additional 28 amino acids. Type 2 is predominantly expressed in adipose tissue, whereas type 3 is expressed in adipocytes and macrophages. Type 1 is widely expressed in all kinds of tissues. Fatty acids and eicosanoids are natural ligands for all isoforms – the TZDs are the best studied synthetic ligands. Activation of PPAR- γ by TZDs induces the expression of a set of genes involved in adipocyte differentiation and lipogenesis, and these effects are thought to be responsible for the insulin sensitizing action of these drugs.

Compared to the other members, only little is known about PPAR- δ . It is expressed in brain, macrophages, lung, adipose tissue and muscle. The natural ligands are prostanoids. There are results suggesting an important role of PPAR- δ in regulation of energy expenditure as well as glucose and lipid metabolism. This highlights the potential use of PPAR- δ modulators as therapeutic agents for diabetes, obesity and atherosclerosis.

1.2. Phytoestrogens – chemical structure, nomenclature and sources

Some naturally occurring compounds present in plants have been found to possess oestrogenic properties by binding to oestrogen receptors. This group of phenolic compounds, been termed *phytoestrogens* is divided into *flavonoids* and *non-flavonoids*. Flavonoids are subdivided into three classes: coumestans, phenylated flavonoids and isoflavons, possessing the most potent oestrogenic activity (Fig.1.1.1). They are low molecular weight hydrophobic compounds, and their watersolubility can be increased by conjugation to glucose, glucuronide or sulfate groups.

Quantification and analysis of phytoestrogens in foodstuffs as well as in pharmacological and toxicological studies are dependent on precise and accurate analytical methods, particularly with regard to the presence of phytoestrogens in only parts per billion to parts per million concentrations in plants, solid and liquid foodstuff as well as in biological matrices such as plasma, serum, urine and faeces. To analyse and quantify phytoestrogens various techniques are used, such as high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [1-3], gas chromatography with mass spectrometric detection (GC-MS) [4-6] and liquid chromatography with mass spectrometric detection (LC-MS) [7, 8]. Recently Immunoessays, using polyclonal antibodies raised against genistein and others have been developed and are equally sensitive, but less time consuming and expensive then the techniques mentioned above [9].

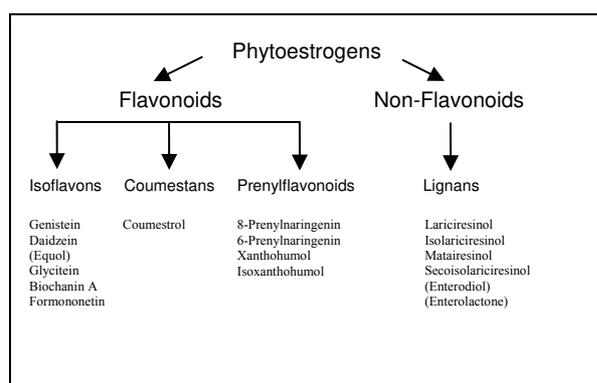


Fig 1.2.1: The relationship between the various groups of phytoestrogens and members of each group.

Natural sources

Phytoestrogens are natural constituents of many plants, seeds and grains. There is considerable variation of phytoestrogen concentrations in different plants. A number of factors can

influence the phytoestrogen content including species, strain differences and environmental conditions [10]. Processing can also alter the phytoestrogen content of foodstuffs. The most prevalent dietary isoflavones include genistein, daidzein, and glycitein. These compounds are primarily found in legumes where they occur as glucoside, while fruits, vegetables, nuts and cereals are all minor sources of isoflavones [11]. Soybeans and soy-based foodstuffs are a particularly rich source of isoflavones, especially genistein and daidzein [12]. In general, commercial processing of soy into food products reduces phytoestrogen concentrations and can alter the chemical form of isoflavones present .

Soybeans have played an integral part in some Eastern cultures (e.g. Japanese and Chinese) as a foodstuff for many centuries. Comparison of estimated dietary isoflavone intakes in Western and Eastern populations illustrate that Eastern populations have a significantly higher intake of phytoestrogens [13].

1.3. Absorption, distribution, metabolism and excretion of phytoestrogens

Absorption

In the natural source – food – phytoestrogens are mainly present as glucosides. Isoflavones are absorbed as aglucones, which are more readily absorbed than the parent glucosides due to their higher hydrophobicity and lower molecular weight. Glucosides of isoflavones themselves have not been identified in plasma, showing that uptake requires hydrolysis of the isoflavone glucosides to their aglucone form [14], although there is debate whether aglucones are more bioavailable than the glucosides. There is evidence that the liver and enterocytes of the human small intestine contain β -glucosidase enzymes capable of efficiently hydrolysing some, but not all, naturally occurring flavone and isoflavone glucosides [15]. Another study also suggested that isoflavone glucosides can be converted to aglucones by enzymes in saliva [16]. Richelle et al showed that plasma concentrations of isoflavones were similar in subjects after consumption of a soy drink which had been enzymatically treated to convert the isoflavone glucosides to their aglucone form, compared to subjects that had ingested the untreated drink [17].

genistein increased dose dependently. Genistein was predominantly (95-99%) present in the conjugated form in plasma but to a much less extent also in tissues. Gender differences in the tissue concentrations of genistein and the proportion of genistein in conjugated form were evident particularly in the liver, thyroid and mammary glands. Only very little genistein was detected in the brain [8]. The following figure shows the plasma and tissue concentration of genistein (Fig 1.3.2).

Pharmacokinetically, plasma genistein concentrations had significantly increased 2 hours after ingestion and reached maximum concentrations 6 hours thereafter. In the same study, plasma concentrations of daidzein peaked at approximately the same time but was lower than genistein [22]. Most isoflavones were recovered in the faeces 2-3 days after ingestion. In a study of Setchell et al the mean plasma half-lives were 7.7 hours, while peak plasma concentrations again were greater for genistein than daidzein at the doses used, suggesting genistein is the more bioavailable isoflavone [23]. The extent of free phytoestrogen available for biological interaction in the plasma is determined by the extent to which they are bound to plasma proteins which renders them unavailable to interact with other molecules, such as oestrogen receptors (ERs).

Tissue	Genistein Concentration pmol/mg (% aglucone)	
	Male	Female
Plasma ^a (adult)	6 $\mu\text{mol/L}$ (<5%)	7.9 $\mu\text{mol/L}$ (<5%)
Plasma ^a (weaning)	1.9 $\mu\text{mol/L}$ (<5%)	2.1 $\mu\text{mol/L}$ (<5%)
Mammary glands	0.8 (24%)	2.4 (49%)
Thyroid	0.4 (25%)	1.2 (18%)
Liver	0.7 (34%)	7.33 (77%)
Brain ^b	Lod	Lod
Prostate	1.1 (45%)	
Testes	0.6 (11%)	
Ovary		1.1 (80%)
Uterus		1.4 (100%)

Fig. 1.3.2: **Plasma and tissue concentrations of genistein in male and female rats.**

Concentrations and percentage unconjugated genistein shown are from animals (n=6) exposed to 500mg genistein/kg diet. Adapted from Chang et al. [8]

Excretion

Excretory routes for phytoestrogens are urine and bile. Several bacterial metabolites of isoflavones have been detected in urine and faeces. Maskarinec et al. compared intake and

excretion of different isoflavones and found that Chinese subjects, having an isoflavone intake of 38 mg/d excreted 307,6 mmol/h via urin, while Caucasian subjects with an daily intake of only 6,9 mg excreted 139 mmol/h [24]. The excretion of daidzein in rats was investigated after a single oral dose of 100 mg daidzein/kg bw [25]. Total urinary excretion accounted for < 10% of the dose. Coldham et al. found, following an oral dose of 14 C-labeled genistein (4 mg/kg bw) to rats of both gender, that 66% and 33% of the dose was excreted in urine and faeces, respectively [26].

1.4. Oestrogen action and receptor mechanism

Oestrogens are hormones of the steroid family involved in various processes regulating development, growth and function in adults. They interact with numerous tissue types in both, males and females. They mainly act through ligand-activated nuclear transcription factors, known as oestrogen receptors (ERs). They – like other steroid hormone receptors – are members of the nuclear receptor family of transcription factors that exhibit common structural domains. If not complexed with *heat shock proteins* (hsp) in the cytoplasm waiting to be activated by a certain ligand, they are predominantly located in the nucleus. Activated dimers show a high binding affinity for specific DNA-binding sites called oestrogen receptor response elements (ERE), which are located in an upstream promotor position of oestrogen sensitive genes (Fig 1.3.1). Probably other proteins, generally referred to as coactivators or codepressors are essential for ER action influencing the level of expression of oestrogen-related genes [27, 28]. The structure of EREs is a 13-base pair inverted repeat motif (2 inverted repeats of AGGTCA or at minimally GGTC A) [29], facilitating the binding of ER units as a homodimer, while sequences flanking the ERE may also play a role in the binding process of ERs. At the same time an alternative way of oestrogen-related gene activation is discussed, using an ERE-independent pathway. ER then interacts with *activating protein 1* (AP-1) and with transcription factor Sp1, causing activation of respective responsive elements [30].

The differential tissue distribution of ER and their variant subtypes may only partly explain the tissue specific effects of oestrogens. Tissue specificity may in fact be attributable to the nature of the dimers formed by receptors and their interaction with accessory proteins. Different groups have found that ERs are able to form certain heterodimers [31-33] that bind

directly to the ERE sequence. In consequence there are at least three possible pathways through which oestrogens may activate target genes by dimerisation.

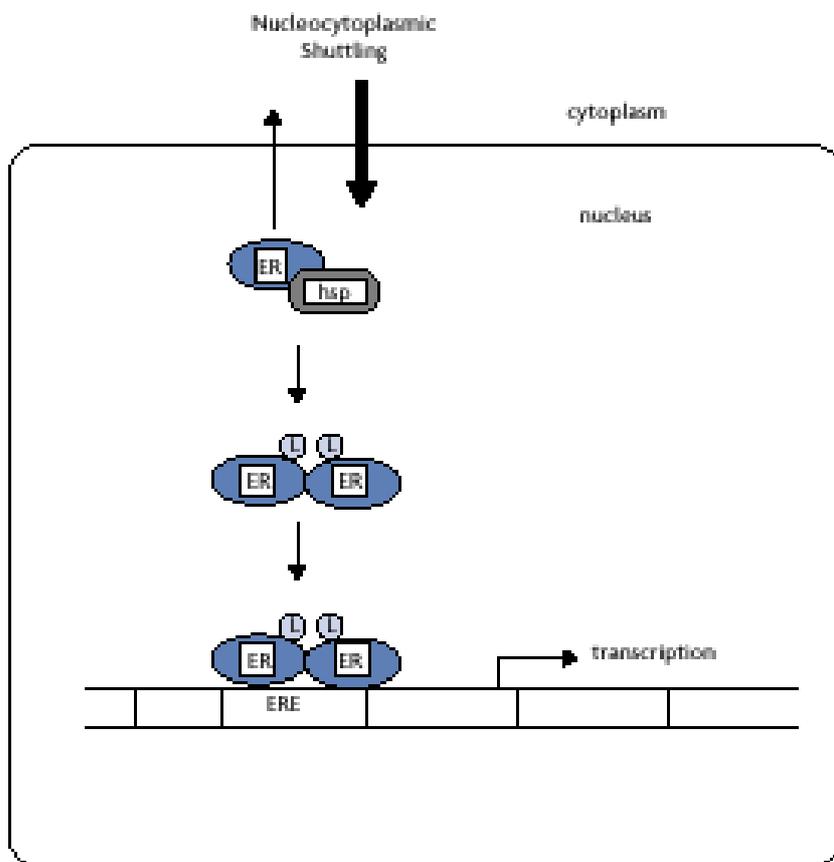


Fig. 1.4.1: Mechanism for the receptor mediated induction of oestrogen responsive genes.

Oestrogen receptors can move between the nucleus and the cytoplasm, but under normal conditions they are predominantly located in the nucleus. Upon entering the cell, oestrogens bind to ERs which are complexed with heat shock proteins (hsp). Upon ligand binding (L), the hsp dissociate from the receptor, allowing two ERs to dimerise and bind to DNA containing the oestrogen responsive elements (ERE) found within genes responsive to oestrogen. Receptor and ERE binding promotes gene transcription and the synthesis of protein which produces an oestrogenic response such as cell proliferation (Adapted from Parker [29]).

Oestrogen receptor subtypes

To date, basically 3 different receptor subtypes are described [34-36]. The ER binds a large number of compounds that exhibit remarkably diverse structural features. In fact, the oestrogen receptor is probably unique among the steroid receptors in its ability to interact with a wide variety of compounds. This is also true for the ER α and ER β subtype.

ER α and ER β subtypes can interact with a wide variety of different compounds although some ligands appear to have different relative affinities for the different subtypes. Showing their very different origin in humans, the gene for ER α is located on chromosome 6 while the one for ER β is located on chromosome 14 [37]. Their very similar protein structure suggests that both receptors may interact with similar response elements. It is known, that ER α and ER β subtypes both, bind oestradiol and other ligands with similar affinity. On the other hand it has been proposed that both receptors act in an antagonistic way. ER β often appears to quench activities up-regulated by ER α , particularly in the context of cell proliferation which has been shown in *in vitro* cell transfection studies by Reynolds et al. [38].

In addition, several authors described further subtypes of ERs, like ER $\beta\Delta 5$ [39], ER β_x [40] and several subtypes of ER α [41-43], but their function is uncertain and needs further investigation.

Tissue distribution of ERs

It does not surprise that expression of ER α and ER β has been found in all major human uterine cell types at every menstrual stage, although variable expression profiles of the two receptor types occur within tissues of the developing human fetus.

In rat and mouse, different regions and cell-populations in the brain, including the Hypothalamus and pituitary gland appear to express either ER α or ER β exclusively, although one minor cell population within the rat pituitary co-expresses both receptors. The different cell types within the rat ovary have also been found to exhibit differences in receptor expression and these may be influenced by the stage of development or maturation of the animal. A list of tissues expressing either both or at least one receptor type is given in figure 1.4.3, showing the complex expression pattern of these two receptors, as well as the different distribution between rodents and humans.

Organ/Tissue	Human		Rodent		
	ER α	ER β	ER α	ER β	ER β 2
Lung	-	✓	-	✓	-
Vascular	✓	✓	-	-	-
Adrenal	✓	-	✓	-	-
Kidney	✓	✓	✓	-	✓
Prostate	-	✓	-	✓	✓
Testes	-	✓	✓	✓	-
Heart	✓	✓	✓	✓	-
Brain	✓	✓	-	✓	✓
Thymus	-	✓	-	✓	-
Breast	✓	✓	-	-	-
Uterus	✓	✓	✓	✓	✓
Endometrium	✓	✓	-	-	-
Vagina	✓	-	-	-	-
Fallopian tube	-	✓	-	-	-
Ovary	✓	✓	✓	✓	✓
Bladder	-	✓	-	✓	-
Epididymus	-	✓	✓	✓	-
Pituitary	-	✓	✓	-	-
Liver	✓	-	✓	✓	✓
Muscle	-	-	✓	✓	✓
Fat	-	-	✓	✓	✓
Gastrointestinal tract	-	✓	-	✓	-
Colon	-	✓	-	✓	-
Small intestine	-	✓	-	✓	-
Bone	✓	✓	✓	✓	✓

Fig. 1.4.3: Tissue distribution of ER subtypes in humans and rodents.

Binding of phytoestrogens to ERs

Fitting very well to the binding site of ER which interacts directly with the ligand and determines its affinity for the receptor, oestradiol shows a high affinity. Because of the flexible character of the binding site of ER and its ability to accommodate a wide range of compounds with structural similarities it was shown that phytoestrogens also bind to these [44-47]. Their affinity for ERs is defined by their biochemical structure (Fig. 1.4.4).

Compound	Binding at ER α (%)	Binding at ER β (%)
Oestradiol ^a	100	100
Coumestrol ^a	34	100
Genistein ^a	0.7	13
Daidzein ^a	0.2	1
8-Prenylnaringenin ^b	10	10

Fig. 1.4.4: The selectivity of oestradiol and phytoestrogen binding to ER α and ER β

The table shows the binding affinities of phytoestrogens to each receptor subtype relative to the binding affinity of oestradiol (arbitrarily set at 100). (a) as referred to Kuiper et al [48]; (b) as referred to Milligan et al [49]

The ringarrangement and the presence and position of chemical groups such as hydroxyl groups [50, 51] play important roles in the structural compatibility. Genistein for example binds to both ERs, which can be explained by the similar size to oestradiol and has hydroxyl groups which are appropriately positioned to fit in the binding pockets (Fig. 1.3.5-1.3.7) [44, 45].

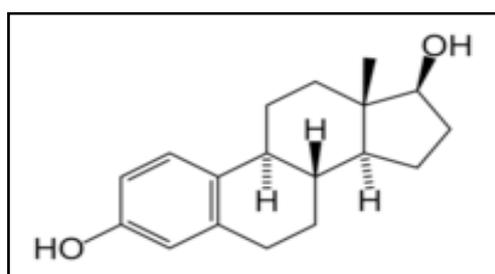


Fig 1.4.5: Beta-Oestradiol

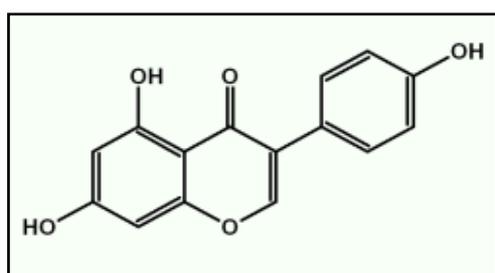


Fig 1.4.6: Genistein

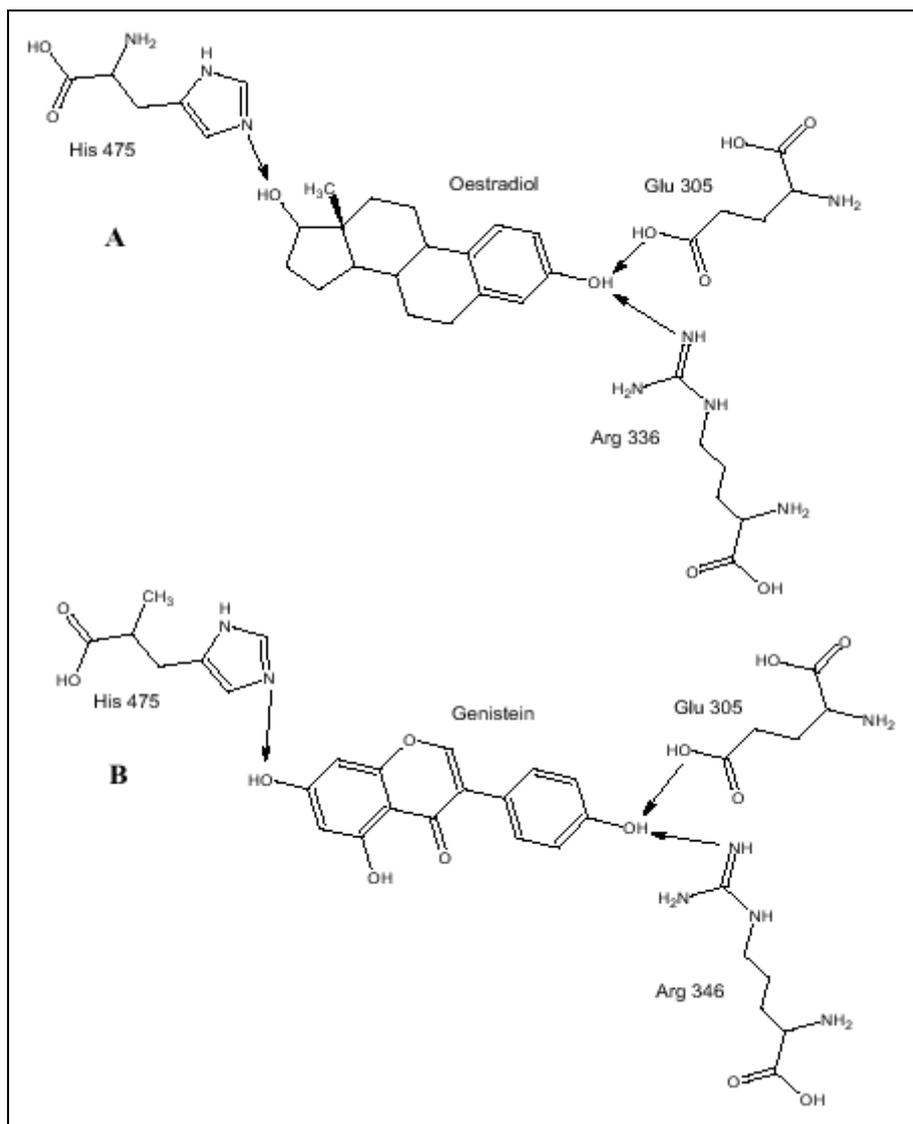


Fig. 1.4.7: Oestradiol and genistein binding to the ligand-binding domain of the oestrogen receptor.

The figure shows selected interactions (marked with arrows) between oestradiol (A) and genistein (B) and specific amino acids in the ligand-binding pocket of ER β .

Binding to ERs, phytoestrogens may act in an activating or blocking way. If phytoestrogens bind to and activate the receptor – similar to oestradiol – this is referred to as *agonist* activity [52]. If it binds to the ER, but blocks further biological response, it is referred to as *antagonist*. If phytoestrogens are able to induce a biological response at levels comparable to these of oestradiol they are called *potent agonists*, in spite of those, which require much higher concentrations to cause a biological response and are deemed *weak agonists*.

The same is true for antagonists. Some phytoestrogens are very weak agonists and by blocking the oestrogen receptor for binding of more powerful ligands they have antagonist activity. These compounds are known as *partial agonists* [53, 54].

It was also predicted that the receptor-bound ligand is completely surrounded by the receptor with minimal exposure to solvent [44].

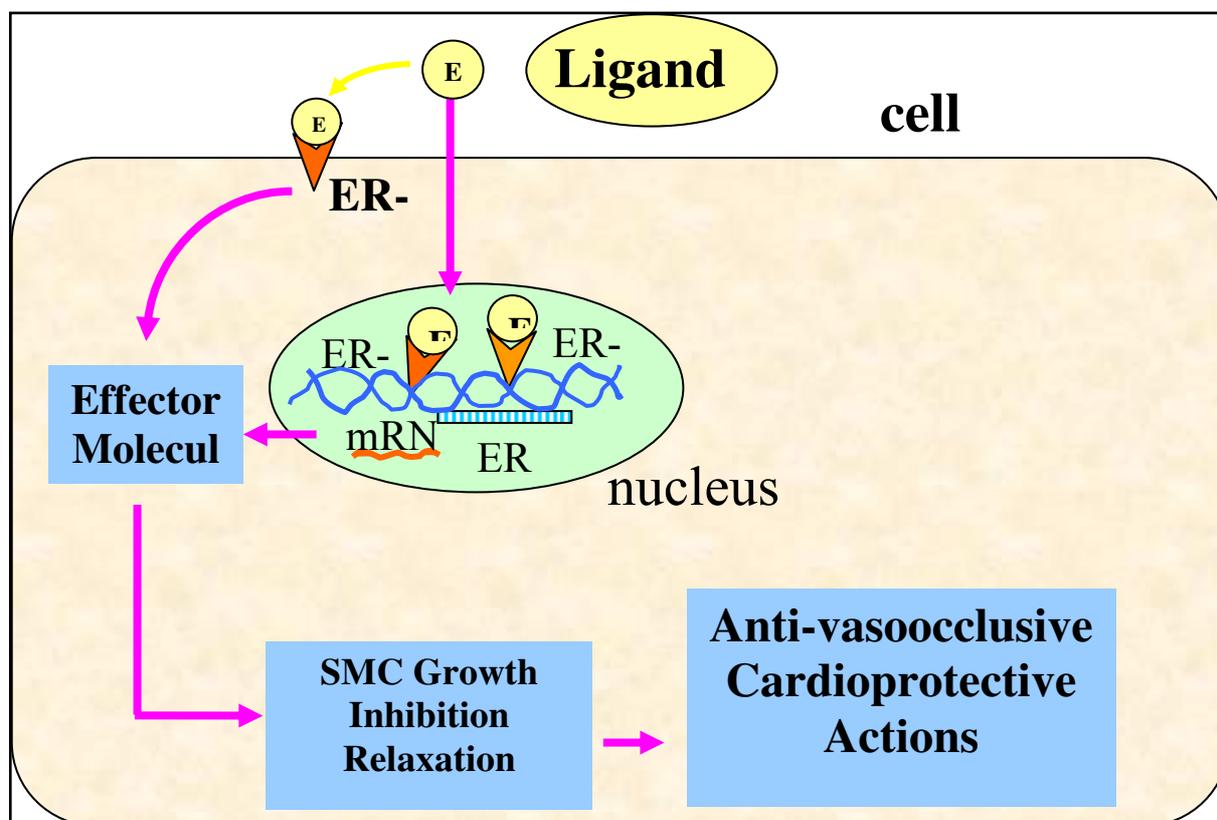


Fig 1.4.8: Biological Effects of Estradiol: the Conventional Mechanism

Apart from the regular ligand-dependent way of activation (Fig. 1.3.8), there is a ligand-independent way by phosphorylation, caused by human epidermal-growth-factor (HEGF), receptor HER2 and insulin. Using the Ras-signal-transduction pathway and the mitogen-activated-protein-kinase (MAPK), they are capable of activating the transcription of ERs-related genes.

Once bound by oestrogens, the ER undergoes a conformational change allowing the receptor to interact with chromatin and to modulate transcription of target genes [55-57].

1.5. Effects mediated by phytoestrogens

Indirect effects of phytoestrogens

Like oestradiol and testosterone, phytoestrogens are circulating bound to *sex-hormone-binding-globulin* (SHBG). In adult women, nearly all oestradiol is associated, either to SHBG (37%), or to serum albumin (61%), while only unbound hormone can be taken up into cells and bind to ERs and there elicit biological effects. Interestingly also testosterone is associated with binding proteins to nearly 100%, 40% is bound to SHBG.

By binding to SHBG, phytoestrogens inhibit binding of oestrogens (and testosterone) to increase the plasma concentration of their unbound form. Although Nagel et al. [58] were able to show that isoflavones bind to SHBG with a 1000-5000-fold lower affinity than oestradiol, this effect may play a certain role. At the same time it has been suggested that phytoestrogens stimulate the synthesis of SHBG and thereby reduce the concentrations of free hormone-levels in the circulation plasma.

Clinical effects

Clinical effects of phytoestrogens are very complex and involve completely different organ systems. It would be very extensive to introduce all known effects of phytoestrogens, especially because they affect dozens of different specialities in the clinical environment. The following enumeration gives an overview of the systems, involved in phytoestrogen effects:

1. fertility and development
2. thyroid gland and thyroid function
3. central nervous and immune systems
4. osteoporosis
5. cancer
6. cardiovascular system

1. fertility and development

Testosterone produced by the fetal testes is mandatory for normal sexual development of the male child. Development of the female is not hormone dependent. Exposure of the male and female fetus to oestrogens or androgens disturbs normal sexual differentiation, although potential effects are different in the male and female. Studies on the effects of phytoestrogens on development and fertility are limited in number and scope [59-62]. In utero effects of phytoestrogens are not published for human studies. It is extremely difficult to examine these potential effects on human development and reproduction for both, practical and ethical

reasons. Hence most of the published research has been conducted in laboratory animals. Experiments in rodents suggest that genistein produces oestrogenic effects in both, male and female rodents but effects may be of more impact in the female rodent [59-62]. Exposure during the perinatal, neonatal or prepubertal stages of development produce the most marked effects. The significance of these effects such as alterations in sex hormone concentrations, advancement of vaginal opening, mammary gland development, irregular oestrus cyclicity and abnormal histology of the reproductive tract to humans is unclear.

2. thyroid gland and thyroid function

Experimental data suggest that high levels of phytoestrogens may have a goitrogenic effect in rodents deficient in dietary iodine. Data from human studies suggest that dietary soy or isoflavones are unlikely to affect thyroid function in normal individuals with adequate iodine intake. In children it may be possible, that the isoflavone component of soy-based infant formula may interfere with the thyroid function [63-65].

3. central nervous and immune systems

In humans, the blood brain barrier is not fully developed at birth and for this reason the CNS may be more accessible to phytoestrogens in utero or at birth. However, few studies have examined the transfer of phytoestrogens from the peripheral blood to the CNS. The published data indicates that transfer across the blood brain barrier in rodents is relatively inefficient (at least in adults), as concentrations of isoflavones in the CNS are several orders of magnitude lower than in peripheral blood [66].

A small number of studies have examined the transfer of phytoestrogens from the peripheral blood to the CNS in rodents, where they possibly may influence behaviour, movement, cognition, pain sensitivity and protect against development of neurodegenerative diseases. Exposure to isoflavones (>200 mg/kg diet) can cause low concentrations of proteins in the CNS, although these effects are not shown by representative studies [66].

In terms of influencing the immune function, oestradiol is known to affect the development and organisation of lymphoid tissues and the activity of various cellular vectors of immune function. It is therefore cogitable that phytoestrogens have comparable effects, because there is evidence that oestrogen receptor activation (also caused by phytoestrogens) can influence lymphoid development [67-70].

Genistein has been shown to inhibit human T-cell proliferation, interleukin production and IL-2 receptor expression *in vitro* [67].

4. *osteoporosis*

Numerous studies have shown, that phytoestrogens have a beneficial role in preventing bone loss following ovariectomy and that they are effective in conserving bone in rodent models of osteoporosis [71-77]. Even in humans, epidemiological studies suggest that intakes of phytoestrogens are associated with higher bone mineral density in populations consuming relatively large amounts of soy. There is a paucity of long-term studies in humans investigating phytoestrogen levels in relation to changes in bone mass. Big long-term studies in humans would be required before the efficacy of phytoestrogen-containing foods or supplements can be confirmed.

5. *cancer*

It is known that lifetime exposure to oestrogen is related to high risks of developing breast cancer [16]. It was suggested that exposure to oestrogen during development or early life may also play an important role in programming hormonal homeostasis and may influence the risk of developing cancer later in life. The epidemiological (limited) evidence suggests a protective role for soy-based food in developing endometrial cancer. Studies looking at phytoestrogens in this context are still pending.

There is no evidence from human studies to attribute the protective effect of fibre, fruit and vegetables against colorectal [78] and stomach cancer [79] to their phytoestrogen content. In addition, there is also little or no evidence to associate phytoestrogen application with a reduced risk of cancer of the ovary, prostate [80] or lung.

6. *cardiovascular system*

The structural similarity of phytoestrogens to oestrogens, which have hypocholesterolaemic effects, and the lower cardiovascular disease mortality rates in populations consuming soy have prompted the suggestion that phytoestrogens are protective against cardiovascular disease [12, 59, 81]. There is evidence from epidemiological studies and intervention trials that diets containing soy or soy protein isolates can have a hypocholesterolaemic effect in humans. Human studies have shown dietary soy has no effect on thrombosis. Only one human study suggests that dietary phytoestrogens may have a beneficial effect on one parameter of atherosclerosis [82].

2. General hypothesis and objectives

Phytoestrogens are plant derived phenolic molecules that structurally resemble estrogens. On the basis of its chemical similarity to estradiol the overwhelming view has been that phytoestrogens may mediate its biological actions via estrogen receptors. This notion is further supported by the fact that phytoestrogens bind to both estrogen receptors α and β , as well as the fact that phytoestrogens like genistein mimic the antimitogenic actions of estradiol on SMC growth. Although these findings suggest that phytoestrogens may mimic the effects of estradiol via ERs, however, recent findings from our group provide evidence that the antimitogenic effects of estradiol, but not genistein are blocked the ER antagonist ICI182780. This led us to hypothesize that the antimitogenic effects of phytoestrogens are mediated via an alternative pathway which does not involve ERs. Because, phytoestrogens have been shown to bind to PPAR receptor, together with the fact that drugs/ligands of PPAR inhibit SMC growth and are vasoprotective, we hypothesize that the antimitogenic actions of phytoestrogens may potentially be mediated via PPAR- γ . In the present study using ER and PPAR antagonists we tested our hypothesis.

3. Material and Methods

3.1. Human Aortic Smooth Muscle Cell Culture (HASMCs)

Human SMCs from adult thoracic aortas (Cascade Biologics, Inc.) were cultured under standard tissue culture conditions (37°C, 5% CO₂) in M231 (Cascade Biologics, Inc, USA) culture medium supplemented with smooth muscle growth supplement (containing 5% v/v FBS, human fibroblast growth factor, human epidermal growth factor and insulin) and antibiotics-antimycotic substances (100 μ g/ml streptomycin, 100 μ g/ml penicillin and 0.025 μ g/ml amphotericin B, Gibco BRL, Paisley, UK). Medium was changed every 2 days and when cells reached subconfluency they were washed twice with HBSS (without Ca²⁺ and Mg²⁺, Bioconcept, Allschwil, CH) and trypsinized in 0.025% v/v Trypsin (Sigma, St. Louis, USA). HASMCs in the third through sixth passages were used for all experiments.

3.2. Cryopreservation of Cells

Subconfluent cells were trypsinized in 0.025% v/v Trypsin and centrifuged 10 min, 1200 rpm at RT. The pellet was resuspended in ice-cold culture medium (2ml/75 cm² flask) containing 7.5% DMSO (Fluka, Buchs, CH) as cryoprotective agent. Aliquots of 500 μ l were gradually frozen to -70°C in cryotubes in a Mr. Frosty box. For long-term storage the cells were kept in liquid nitrogen.

3.3. Cell Thawing

The cryotubes containing the frozen cells were rapidly thawed under warm water. The content was transferred to a tube and 14 ml of fresh growth medium were slowly added. The cells were then plated in 75 cm² culture flasks (Gibco BRL, Paisley, UK) and incubated under standard tissue culture conditions.

3.4. DNA Synthesis

H-thymidine (specific activity 11.8 Ci/mmol, ICN Biomedicals) incorporation and cell number studies were conducted to investigate the effects of phytoestrogens on mitogen-induced DNA synthesis and cell proliferation, respectively.

HASMCs were plated in 24 well tissue culture dishes (1x10⁴ cells/well) and allowed to grow subconfluence in DMEM/F12 (phenol-red free, Gibco BRL, Paisley, UK) containing 10% fetal calf serum (steroid free and delipidated, Gibco BRL, Paisley, UK) under standard tissue culture conditions. The cells were then growth-arrested by feeding DMEM (phenol red free) containing 0.4% albumin for 48 hours. For DNA synthesis, growth was initiated by treating growth arrested cells for 20 hours with DMEM containing 2.5% FCS (fetal calf serum) and containing or lacking genistein (Sigma Chemicals, St. Louis, USA) or rosiglitazone or various phytoestrogens (daidzein, equol, resveratrol) with or without the oestrogen receptor antagonist ICI 182,780 (in all experiments 2 μ mol/l, Tocris, Bristol, UK) and PPAR- γ antagonist GW 9662 (in all experiments 2 μ mol/l, Sigma). After 20 hours of incubation the treatments were repeated with freshly prepared solutions but supplemented with H-thymidine for an additional 4 hours. The experiments were terminated by washing the cells twice with Dulbecco's phosphate buffered saline and twice with ice-cold trichloroacetic acid (10%). The precipitate

was solubilized in 500 μ l of 0.3 N NaOH and 0.1% sodium dodecylsulfate (Sigma Chemicals, St. Louis, USA) after incubation at 50°C for 2 hours. Aliquots from 4 wells for each treatment with 10 ml scintillation fluid were counted in a liquid scintillation counter.

3.5. Collagen Synthesis

Confluent monolayers of HASMCs growth arrested for 48 hrs were treated in 24-well plates for 36 hrs with DMEM supplemented with 2.5% FCS plus L-[3 H]proline (1 μ Ci/ml) in the presence or absence of the test agents. Following treatment, the experiment was terminated by washing the cells twice with PBS and twice with ice-cold 10% TCA. The precipitate was solubilized in 500 μ l of 0.3 N NaOH and 0.1% SDS (50°C for 2 hrs). The samples were subsequently neutralized with 100 μ l 5N HCL and aliquots from 4 wells for each treatment were counted in a liquid scintillation counter. To ensure that the inhibitory effects of the experimental agents on collagen synthesis were not due to changes in cell number, the experiments were conducted in confluent monolayers of cells in which changes in cell number were precluded. Additionally, cell counting was performed in cells treated in parallel with the cells used for the collagen synthesis studies, and the data were normalized to cell number.

3.6. Cell number

HASMCs were plated in 24 well plates (5×10^3 cells/well) and allowed to attach overnight. Cells were growth arrested as described above in DNS synthesis for 48 hours and subsequently treated every 24 hours for 4 days. On day 5 the cells were harvested by trypsinization and counted in a Coulter Counter.

3.7. Statistical Analysis

Data was analyzed with StatView using ANOVA and statistical significance ($p < 0.05$) was calculated using Fisher's Least Significant Difference Test. A value of $p < 0.05$ was considered to be statistically significant.

4. Results

All figures are based on mean values and standard deviation of three separate measurements. A value of $p < 0.05$ was considered to be statistically significant. As described above all experiments were performed on human smooth muscle cells (HSMCs) under standard conditions. All values are compared to values of untreated SMCs which served as control.

4.1. Effects of genistein on DNA synthesis

To describe the effects of genistein on DNA synthesis, cell cultures of HSMCs were treated with genistein in different concentrations. DNA synthesis was measured as percentage of DNA synthesis rate of untreated cell cultures of HSMCs which served as control (data not shown).

If PPAR- γ receptor was blocked using GW9662 receptor blocker, DNA synthesis remains stable on levels around 100% compared to control (bright line, figure 4.1.1).

Unblocked cells show a drop of DNA synthesis even treated with low levels of genistein. With increasing levels of genistein the DNA synthesis decreases continuously to levels around 25% treated with a maximum concentration of 100 $\mu\text{mol/L}$ genistein (darkline, figure 4.1.1).

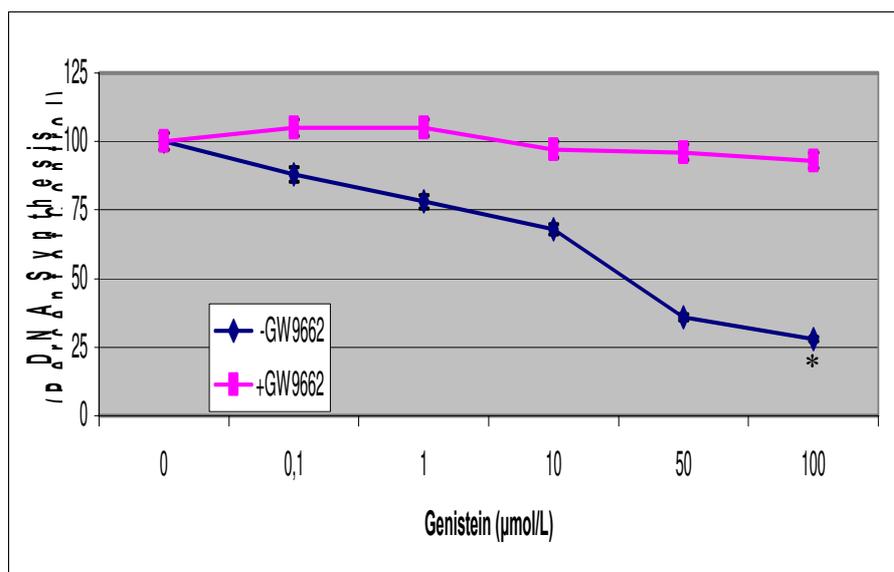


Figure 4.1.1: DNA synthesis; * $p < 0,01$

Figure 4.1.2 shows DNA synthesis rates of SMCs treated with ER-blocker ICI18280 (bright line, figure 4.1.2.) compared to cells with normal ER-status (dark line, figure 4.1.2).

Both lines show a decrease of DNA synthesis depending on different levels of genistein. DNA synthesis decreases with increasing levels of genistein to a minimum of around 25% in cells treated with a maximum of 100 µmol/L genistein not dependent on ER blocker ICI182780.

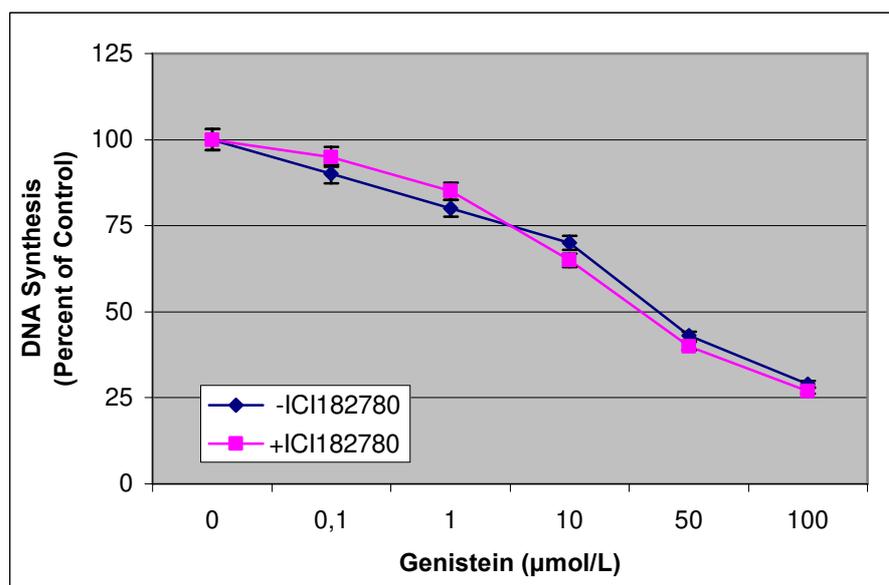


Figure 4.1.2: DNA synthesis

4.2. Effects of genistein on collagen synthesis

To determine the effects of different genistein levels on collagen synthesis SMCs were either treated with PPAR- γ receptor blocker GW9662 or left unblocked. Unblocked cells showed decreasing collagen synthesis depending on increasing levels of genistein to a minimum of around 25% collagen synthesis at a maximum level of 100 $\mu\text{mol/L}$ genistein.

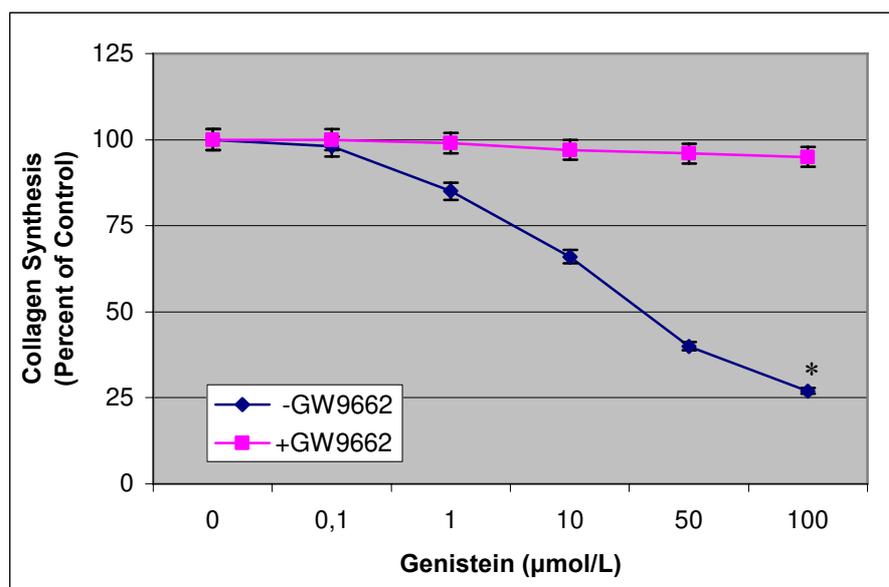


Figure 4.2.1: Collagen synthesis; * $p < 0,01$

Figure 4.2.2 shows SMCs either blocked with ER blocker ICI182780 or left unblocked. Both lines show a decrease of DNA synthesis depending on different levels of genistein. DNA synthesis decreases with increasing levels of genistein to a minimum of around 25% in cells treated with a maximum of 100 $\mu\text{mol/L}$ genistein independent from treatment with ER blocker ICI182780.

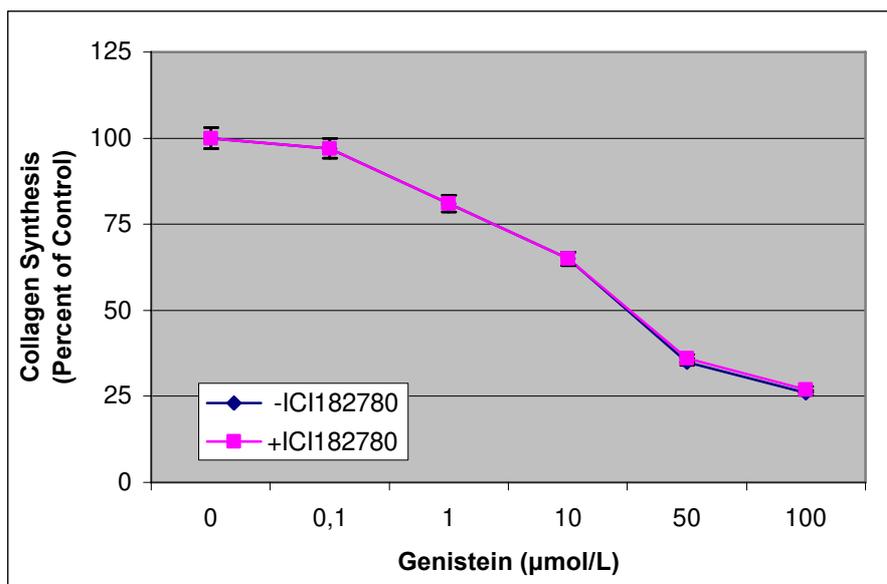


Figure 4.2.2: Collagen synthesis

4.3. Effects of genistein on SMC proliferation

To investigate the effects of different genistein levels on cell numbers SMCs were again treated with PPAR- γ receptor blocker GW9662 or left unblocked. Unblocked cells showed decreased cell numbers depending on increasing levels of genistein treatment to a minimum of around 25% at a maximum level of 100 µmol/L genistein.

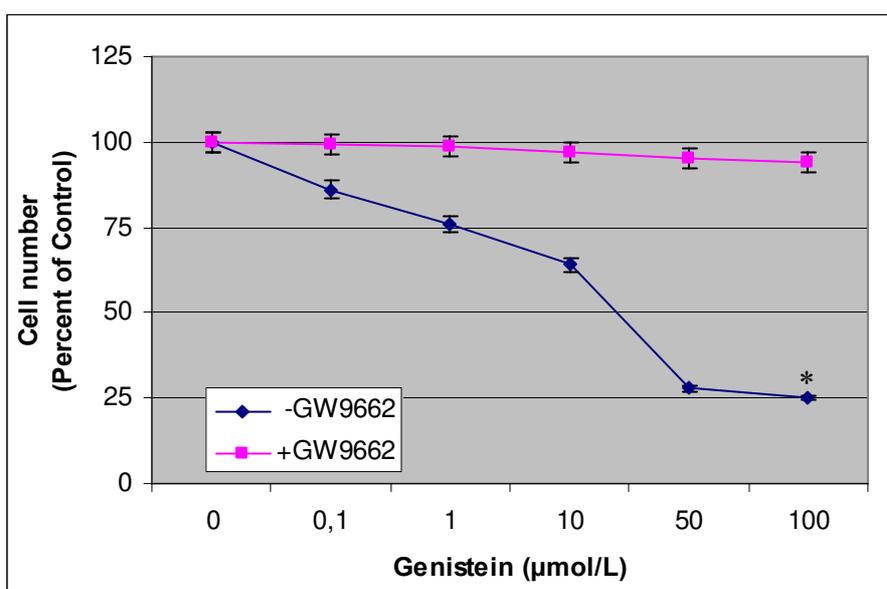


Figure 4.3.1: Cell number; *p<0,01

Both, ER-blocked SMCs and unblocked SMCs showed decreasing cell numbers depending on different levels of genistein. The cell number decreases with increasing levels of genistein to a minimum of around 25% in cells treated with a maximum of 100 $\mu\text{mol/L}$ genistein not dependent on treatment with ER blocker ICI182780.

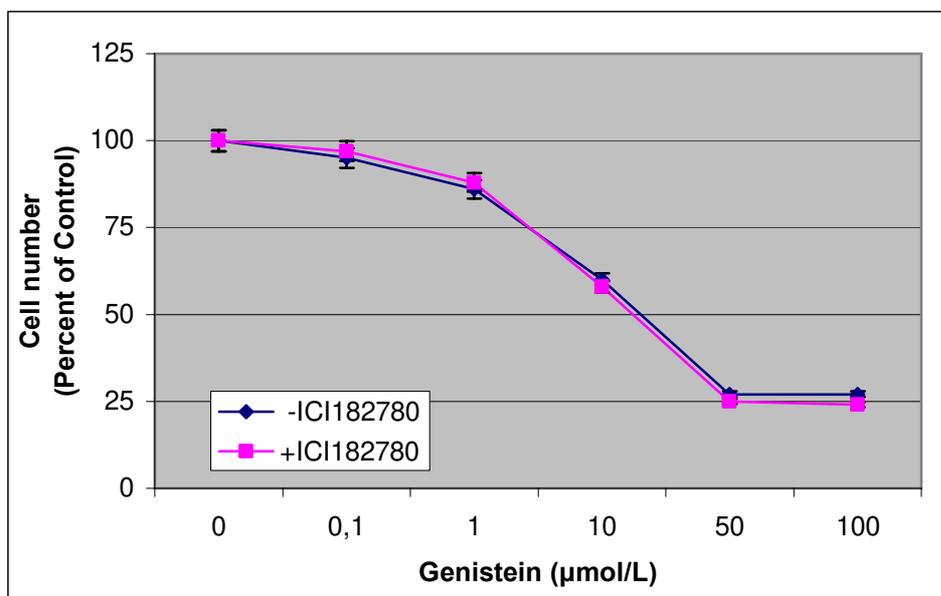


Figure 4.3.2: Cell number

4.4. The effect of Rosiglitazone and Genistein on SMC growth via PPAR- γ

To show the effects of the best studied PPAR- γ agonist Rosiglitazone [83] on cell numbers SMCs were again blocked with PPAR- γ receptor blocker GW9662 or left unblocked. Unblocked cells did not show a significant decrease of cell numbers even under increasing levels of Rosiglitazone up to 100 $\mu\text{mol/L}$. Blocked SMCs instead show a significant decrease of cell numbers to a minimum of around 60% depending on the increase of Rosiglitazone levels to a maximum of 100 $\mu\text{mol/L}$.

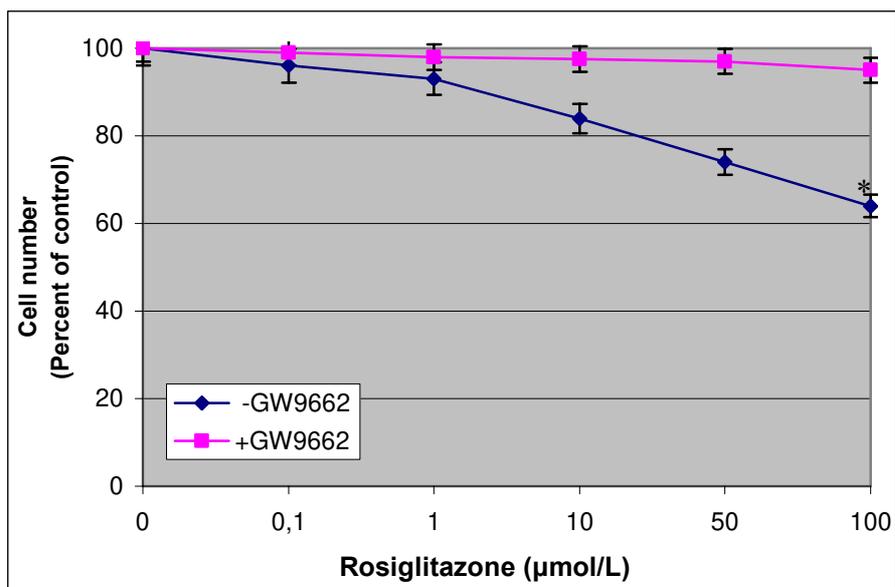


Figure 4.4.1: Cell number; *p<0,01

Comparing the effects of Rosiglitazone and genistein on cell numbers Rosiglitazone in a dose of 100 µmol/L leads to a decrease of cell numbers to a minimum of around 60%.

A treatment with 100 µmol/L genistein lowered the cell number to levels around 25%. Notice the drop of cell numbers in the genistein treatment when dose is raised from 10 to 50 µmol/L. If genistein dose is again raised from 50 to 100 µmol/L cell numbers nearly remain stable at levels of 25%.

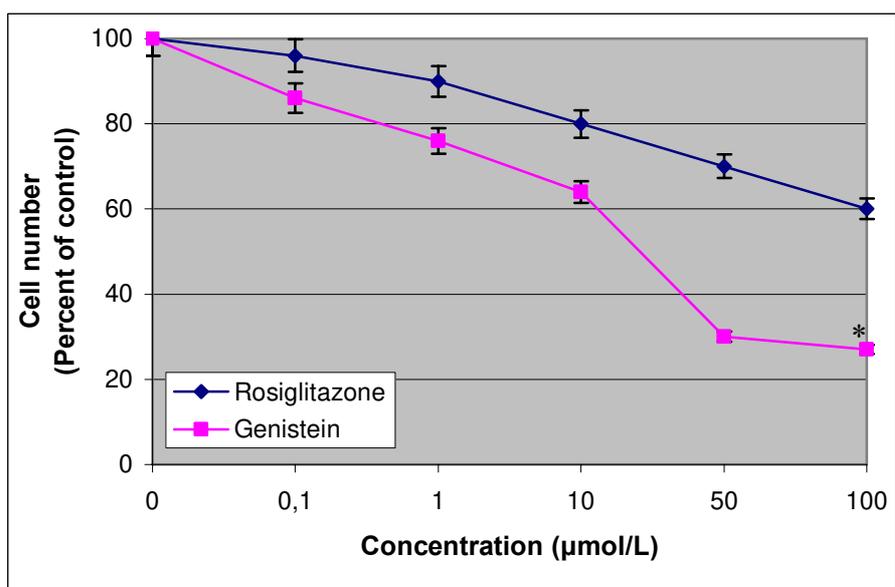


Figure 4.4.2: Cell number; *p<0,01

4.5. Antiproliferative Actions of Estradiol, Rosiglitazone and phytoestrogens

Looking at the effects of treatment with estradiol, rosiglitazone and genistein (each 10 $\mu\text{mol/l}$ in all experiments) on cell numbers in SMCs either blocked with GW9662 or not, oestradiol had the lowest effect on cell numbers independent from treatment with PPAR- γ receptor blocker GW9662.

Measured four days after treatment rosiglitazone shows the most significant effect with a cell number of around 75% in unblocked and around 97% in blocked SMCs.

Compared to rosiglitazone, genistein treatment lead to cell numbers of around 62% in unblocked and around 90% in blocked cells.

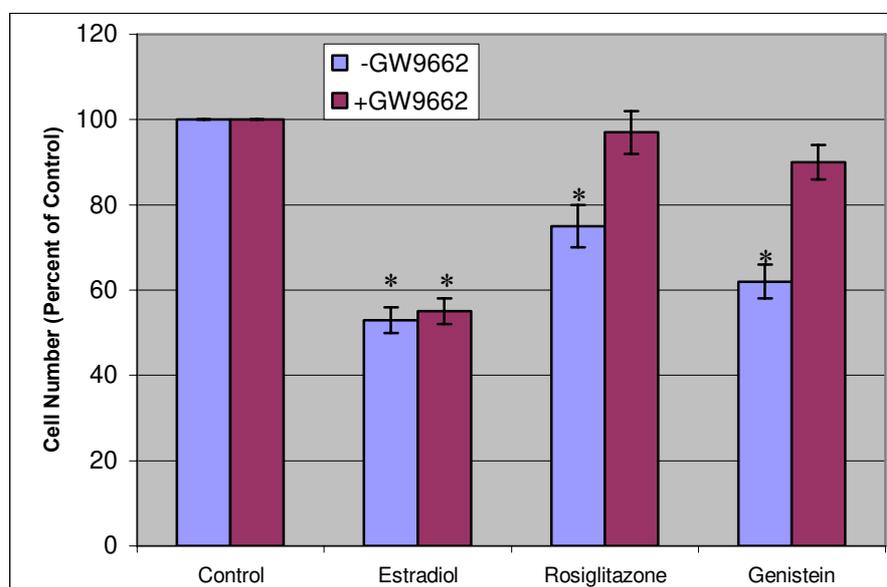


Figure 4.5.1: Cell number; * $p < 0,01$ (compared to control)

To demonstrate the effects of different phytoestrogens on DNA synthesis, receptor blocker GW9662 was again used to block PPAR- γ -receptor in SMCs. Measurements were performed four days after treatment. While DNA synthesis in control group remained 100% in blocked and unblocked cells, it decreased under treatment of different phytoestrogens whether SMCs were blocked or not. Apart from cells treated with Equol, DNA-synthesis dropped to higher extent when PPAR- γ receptor was left unblocked. Treated with Equol, DNA synthesis remained stable at a level of 80% compared to control in both, blocked and unblocked cells. Genistein treatment of cells with unblocked PPAR- γ -receptor showed the highest level of decrease with a value of 40% compared to control.

In general, a phytoestrogen treatment induced a decrease of DNA synthesis in SMCs to a level of around 60% (compared to control), while an additional treatment with PPAR- γ -receptor blocker GW9662 lowered the DNA synthesis rate to levels of around 80%.

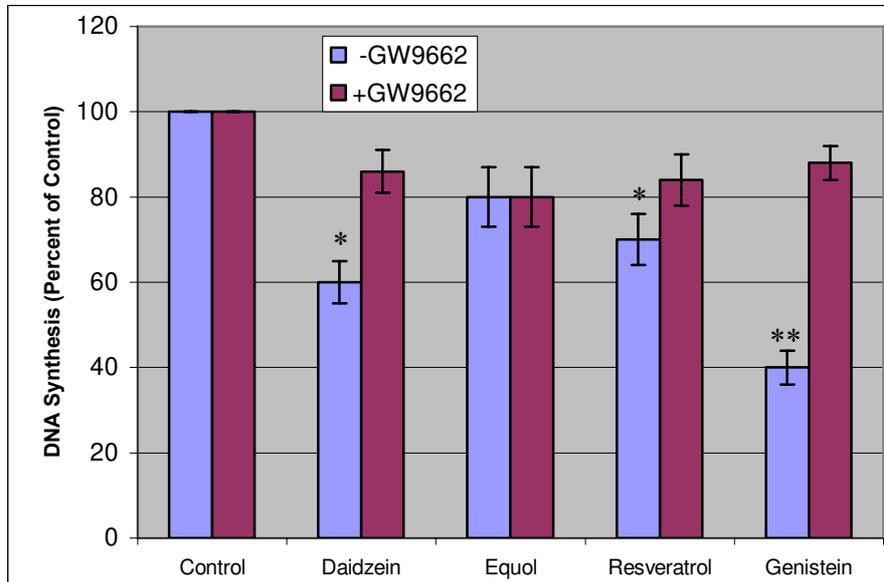


Figure 4.5.2: DNA Synthesis; * $p < 0,05$; ** $p < 0,01$

5. Discussion

5.1. Clinical background

Most Western countries face high and increasing rates of cardiovascular disease. Each year, heart disease kills more people than cancer. Diseases of the heart alone account for 30% of all deaths, with other diseases of the cardiovascular system causing substantial further death and disability. It is the number one cause of death and disability in the United States and most European countries. Studies showed vascular injury accumulates from adolescence, making primary prevention efforts necessary prior to its initiation. By the time the heart problems are detected, the underlying cause (atherosclerosis) is usually quite advanced, having progressed for decades. There is therefore increased emphasis on preventing atherosclerosis by modifying risk factors, such as healthy eating, exercise and avoidance of smoking.

Cardiovascular disease (CVD) has a very high incidence in both, man and woman [84-87]. Relevant factors for the genesis of CVD are diabetes mellitus, high blood pressure, high levels of lipoproteins (low-density lipoproteins, LDL) and adipositas, smoking and age – to name some known causes (Fig. 5.1.1) [88-92]. CVD can cause severe effects to the human body like cerebral insults or heart attacks. It further causes peripheral atherosclerosis which can lead to surgical intervention or even limb amputation. This makes CVD to a major disease in the western world, a major cause of death and a significant financial factor in the national health system [93, 94]. The pathogenesis of atherosclerosis is based on the development of changes in the vascular system. High amounts of LDL stick to sights of damage at the vessel wall structure, causing what is called plaques. These plaques lower the diameter of the vessel and therefore the blood flow through the constriction, leading to a decreased blood supply in the periphery. Proliferation of SMCs from the vessel wall and recruitment of inflammatory cells to these sights of plaque-formation can then produce vasoocclusive diameters by interacting with thrombocytes and other inflammatory cells. This acute incident can cause a complete blood flow breakdown situation such as cerebral or coronary arteries – a heart attack or cerebral insult occurs.

Interestingly, although CVD is the number one cause of death for individuals in modern society, a majority of young people are unconcerned about the risk for development of this disease. Atherosclerosis is a process that develops over decades and is often silent until an acute event (heart attack) develops in later life. Intimal lesions appear in all the aortas and

more than half of the right coronary arteries of youths aged 15–19 years. However, most adolescents are more concerned about other risks such as HIV, accidents, and cancer than cardiovascular disease. This is extremely important considering that 1 in 3 people will die from complications attributable to atherosclerosis.

- Non-modifiable Risk Factors
 - Age
 - Gender, men under age 64 are much more likely to die of coronary heart disease than women, although the gender difference declines with age.^[2] (The gender difference is less pronounced in blacks than in whites, but it is still significant^[3])
 - Genetic factors/Family history of cardiovascular disease

- Modifiable Risk Factors
 - Tobacco smoking
 - Insulin resistance & Diabetes mellitus
 - Hypercholesterolemia (elevated cholesterol levels) and abnormal lipoprotein particle profile (cholesterol subtypes)
 - Obesity, especially central or male-type obesity; apart from being linked to diabetes, this form of obesity independently increases cardiovascular risk, presumably by inducing an inflammatory and procoagulant state
 - High blood pressure
 - Elevated heart rate^[4]
 - Physical inactivity/Sedentary lifestyle
 - Absence of key nutritional elements, such as omega-3 fatty acids and polyphenol antioxidants
 - Exposure to high levels of environmental noise
 - Stress
 - Depression
 - Periodontal disease

Fig. 5.1.1 risk factors which associate with (but are not all causes of) various forms of cardiovascular disease

It is well known, that – compared to man of the same age – women younger than 45 years are somehow “more protected” against CVD [95]. After menopausal changes in the hormonal system – either by surgical intervention or by normal aging – the risk of CVD increases rapidly in females [96-100]. This is caused by a shift of the lipidprotein profile from protective high-density lipoproteins (HDL) to low-density lipoproteins (LDL), which is a major cause of atherosclerosis and therefore CVD. LDL receptor numbers decrease which leads to a low level of LDL katabolism [101]. It is well known, that oestrogen replacement therapy (HRT) can protect the cardiovascular system from these effects [102-105]. Some animal models show that further effects of oestrogens help to lower the risk of CVD: Both, blood vessels and

heart endothelia can express oestrogen receptors and thereby influence vessel tonus and cell-adhesion to the vessel wall which are important factors for the development of plaques causing atherosclerosis [106]. Studies show a decreased risk for CVD of 50% in female patients with HRT [104, 107] while other studies discuss these effects controversially. One major problem is the fact, that apart from animal experiments showing cardioprotective effects of oestrogens, these results are not acknowledged by some other big studies [108]. The use of different types of oestrogens in studies could possibly explain the different results.

Apart from the effects to the cardiovascular system there are severe side-effects of HRT such as increased risk for breastcancer, thrombosis and strokes beside many more [109]. It is therefore very important to find a treatment for postmenopausal women which provides a good cardioprotective effect and does not cause the unwanted side-effects of oestrogen. A possible target drug in this struggle are phytoestrogens such as genistein and others [110]. Different studies show good cardiovascular-protective effects [81, 110-119].

5.2. Effects via PPAR- γ receptor

Although phytoestrogens are molecules not produced by the human body itself, different studies showed a binding to both, circulating globulins and oestrogen receptors [44-47]. Because of this very similar chemical structure and the binding to oestrogen specific sights it seems only logic that oestrogen receptors are the main mediators of effects caused by phytoestrogens. Our in vitro experiments show for the first time that blocking of oestrogen receptors does not lower certain effects of genistein. This means that phytoestrogens must bind to some other receptor and mediate their effect not only via oestrogen receptors. Focussing on certain pathways which are relevant for potential antiatherosclerotic effects of phytoestrogens, our experiments describe SMC proliferation parameters and collagen-synthesis parameters.

The role of cell proliferation on atherosclerosis with respect to our results

Plaques, the morphologic visible form of atherosclerosis consist of different fractions. Main “building modules” of these intravascular cloggings are circulating cells and cells from the vessel wall to where the plaques are attached. Growing in the intima layer of the vessel wall smooth muscle cells proliferate into the plaques and thereby serve as material for plaque-growing

processes. A low level of cell proliferation in terms of low mitosis rate of these SMCs would lead to a decreased rate of plaques-formation and would therefore protect the vessel against atherosclerosis – one important cause of CVD. This is why we measured the proliferation rate of SMCs under the influence of genistein.

Analyzing our results from measurements of cell numbers and DNA synthesis – both factors indicating the rate of cell proliferation – one can see that genistein can lower DNA synthesis rates and cell numbers in SMC culture with increasing levels of genistein (Fig. 4.1.1 and 4.3.1, each $p < 0,01$). Around a level of 10-50 $\mu\text{mol/l}$ genistein, cell numbers and DNA synthesis rates drop down, indicating an optimal dosage in this concentration range. If PPAR- γ receptor is now blocked by inhibiting effects of GW9662, DNA synthesis and cell number are stable suggesting that the antimitogenic effects are mediated via PPAR- γ receptor only.

If oestrogen receptors are blocked as done in separate experiments, the effects of genistein on cell number and DNA synthesis are not altered. There is no significant difference in the decrease of cell numbers and DNA synthesis under blocked ER showing that these effects are certainly not only mediated via the ER receptor pathway.

Summarising it was shown that the effects of genistein on cell proliferation and DNA synthesis are mediated via PPAR- γ receptor and not only via ER. Concerning the dosage of genistein the optimal amount has to be discussed. In drug treatments the optimal dose describes the maximum of wanted effects causing a relative minimum of unwanted effects. From these results the optimal dose would be between 10-50 $\mu\text{mol/l}$. In our data we can find a “drop-zone” between these dosages. Our results acknowledge the already known protective effects of genistein on atherosclerosis. They show that these effects are not mediated via ER but via PPAR- γ receptor only.

The role of collagen synthesis on atherosclerosis with respect to our results

Another important factor are proteins expressed by plaques-forming cells such as SMCs. The most important extracellular protein for stable matrices is collagen. The chains can form stable networks of fibres leading to a rigid and lasting mass (plaques), which can cause vessel tightening as seen in the process of atherosclerosis. Because of this fact, we analyzed the amount of collagen synthesis by SMCs under the influence of genistein.

If ER is left unblocked, genistein can lower the level of collagen synthesis in SMCs to a level of 25% as seen in figure 4.2.2. The effect of genistein is not influenced if ERs are blocked indicating an effect not depending on ERs. Genistein works in a dose-dependent manner, but

– again – shows a relevant drop of collagen synthesis levels in a dose of 10-50 $\mu\text{mol/l}$ as seen before.

Focussing on figure 4.2.1 where PPAR- γ receptors of SMCs were blocked one can clearly see that the effect on collagen synthesis disappears when PPAR- γ receptors are blocked. This proves, that collagen synthesis, too, depends on the presence of unblocked PPAR- γ receptors but is not dependent on the presence of oestrogen receptors. Most effective levels of genistein are comparable to the other results with an optimal dose of 10-50 $\mu\text{mol/l}$.

Rosiglitazone and genistein – same efficiency?

Rosiglitazone (Fig. 5.2.1) is an oral drug that reduces the amount of sugar in the blood. It is used for treating patients with type 2 diabetes and belongs to a class of anti-diabetic drugs called thiazolidinediones. Member of this class is also pioglitazone. They are marketed by another pharmaceutical company, both as a standalone preparation and in combination with medication from the metformin-family. Rosiglitazone often is referred to as an "insulin sensitizer" because it attaches to the insulin receptors on cells throughout the body and causes the cells to become more sensitive (more responsive) to insulin and remove more glucose from the blood. At least some insulin must be produced by the pancreas in order for rosiglitazone to work. Rosiglitazone was approved by the FDA in 1999. Like other thiazolidinediones, its mechanism of action is by activating the intracellular receptor class of peroxisome proliferator-activated receptors, specifically PPAR- γ . Rosiglitazone is a pure ligand of PPAR γ , and has no PPAR α -binding action.

In our experiments we evaluated the effect of rosiglitazone on cell numbers (anti-proliferative effect) and compared the efficiency to genistein. If SMCs are treated with rosiglitazone cell numbers decrease in a dose dependant manner to a level of 60%. If PPAR- γ receptors are blocked, cell numbers remain stable at levels around 100%, confirming that rosiglitazone mediates its effects via this receptor.

If SMCs of the same source are treated with genistein and rosiglitazone and cell numbers are measured, both treatments lead to decreased cell numbers. As mentioned above rosiglitazone can lower cell numbers of SMCs to a level of 60%. Under genistein treatment using the same concentration the numbers of SMCs decrease to levels of around 25%. This proves that both drugs can diminish the rate of cell proliferation, but genistein has a much more powerful effect in our experiments. In genistein treatment we again find the drop in cell numbers in levels of 10-50 $\mu\text{mol/l}$ genistein, while rosiglitazone shows a stable decrement of cell numbers with

increasing dosages. Summarizing we were able to show, that both, genistein and rosiglitazone mediate their anti-proliferative-effects via PPAR- γ receptors. The effects of genistein-treatments are superior to the effects of rosiglitazone-treatments.

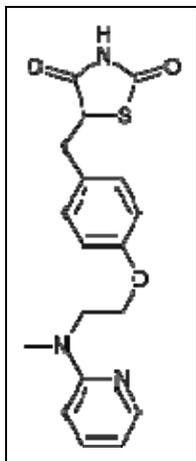


Fig. 5.2.1 chemical structure of Rosiglitazone

Estradiol, rosiglitazone and phytoestrogens

One additional experiment was performed to proof, whether differences in the antiproliferative effects of different PPAR- γ receptor ligands can be quantified or not. For this purpose SMC cultures were treated with estradiol, rosiglitazone and genistein. Cell numbers were measured as percentage compared to a SMC culture without any treatment. Drug levels were equal throughout all treatments with 50 $\mu\text{mol/l}$. While untreated cell numbers stayed stable with (defining) 100%, SMCs treated with estradiol, rosiglitazone and genistein showed decreased cell numbers in all measurements. Estradiol is the most effective anti-proliferative drug, while genistein and rosiglitazone have comparable effects (Fig. 4.5.1). While blocking of PPAR- γ receptors does not have any additional effect in SMCs treated with estradiol, it certainly has in cells treated with rosiglitazone and genistein. This proves, that the antiproliferative effects are mainly mediated via PPAR- γ receptor-dependent pathways. Our results further demonstrate, that genistein – although not as powerful as estradiol – is superior to rosiglitazone by the means of anti-proliferative effects.

Measuring the DNA-synthesis rate in SMC cultures, cells were treated with different phytoestrogens (same dosage of 50 $\mu\text{mol/l}$) to compare the effects with blocked and unblocked PPAR- γ receptors. Our results demonstrate that daidzein, resveratrol and genistein work via PPAR- γ receptors: if these receptors are blocked, they show no significant effect on DNA-synthesis. Under unblocked condition genistein again has the most significant effect,

lowering DNA synthesis rates to levels of 40% compared to control. This again demonstrates the powerful efficiency of this drug (Fig. 4.5.2).

The “perfect” target drug

From the results shown above some properties of the target drug in the struggle to fight atherosclerosis can be characterized. The drug needs to slow down up-regulated SMC proliferation into the plaques and decrease the rate of collagen synthesis. We were able to demonstrate, that genistein has a dose-dependent property to lower the rate of SMC proliferation. We were further able to show for the first time, that this effect is certainly mediated via the PPAR- γ receptor. Also mediated via this receptor type, genistein can decrease the rate of collagen synthesis in SMCs. Thereby it unites two important properties to decrease the risk of plaques-formation and risk of atherosclerosis and CVD. Compared to other known molecules genistein has a significant higher efficiency apart from oestradiol. Oestradiol itself has numerous side effects and is hence not the perfect drug in the treatment of atherosclerosis. We were also able to demonstrate throughout all our experiments, that there must be something like an optimal dosage by the means of “highest effect at lowest rate”. This “watershed” is around levels of 10-50 $\mu\text{mol/l}$. All the demonstrated experiments are in vitro experiments, why the treatment levels cannot be transferred to in vivo setting.

Critics

The experiments shown in this paper are based on ex-vivo models. We investigated certain effects of genistein on SMC-cultures. The results mentioned above are a good basis to discuss effects of genistein administered to the human body. One problem remains the different situation in vivo, where resorption in the digestive system, administration in the compartments of the body and so on could lead to different results in SMCs. It is further possible, that other cell types apart from SMCs are involved in the effects on genistein which can cause a higher or lower effect compared to the cell culture. To investigate the effects of genistein on SMCs in a complex system like the human body, animal experiments can help to answer these questions. As the effects of genistein on SMCs are very much dependent on the amounts of genistein molecules in the extracellular milieu it needs to be investigated which amounts of genistein are present at the sights of SMC-growth in in-vivo models.

Genistein is only one molecule of the family of phytoestrogens. From the experiments shown in this work it is not possible to conclude that other phytoestrogens act in a similar way. It is possible that different distribution and metabolism influences the effects of other phytoestrogens. Further experiments need to be done to study their differences and similarities. Our results prove that the measured parameters are mediated via PPAR- γ receptors, because by blocking this receptor and leaving the ERs unblocked at the same time, we were not able to measure the effects. ERs obviously do not act like key receptors in transmitting these effects, but are more or less irrelevant. This conclusion can only be made for genistein while other phytoestrogens need to be studied for their interaction with the PPAR- γ receptor.

5.3. Perspectives

As already described in the introduction, phytoestrogens are oestrogen-like molecules, binding to estrogen receptors and others. Their side effects are minimal and they show good impact in totally different areas like fertility and development [59-62], thyroid function [63-65], central nervous and immune system [67-70], osteoporosis [71-77] and the cardiovascular system [81, 111-119]. This shows the immense complexity in the effects of this group of molecules. Their presence in food (Fig 5.4.1) makes them available to nearly every human being to high degrees. After intake they show high levels of absorption and distribution to different tissues. Their metabolism and excretion makes them low in risk and practical for common use. Their oestrogen-like effects are well known, also it is still not clear, whether they use similar pathways or not.

In patients with atherosclerosis after menopausal changes, hormone replacement therapy is well known to lower the risk of CVD [102, 103], although these results are discussed controversially.

As mentioned above it is therefore very important to invent a treatment for postmenopausal women which provides a good cardioprotective effect and does not cause the unwanted side-effects of oestrogen. We were able to show that one target drug in this struggle is genistein, a phytoestrogen which leads to a decreased rate of SMC proliferation and DNA-synthesis as well as a decreased collagen synthesis – both mandatory factors in the genesis of atherosclerosis. The easy oral application of genistein and other phytoestrogens makes them a practical target-drug to fight atherosclerosis. As discussed above, more animal studies are

needed to investigate and specify certain effects of genistein on different organs to make it a useful and safe drug. With respect to CVD and atherosclerosis it is possible that phytoestrogens may replace oestrogens in the therapy of postmenopausal women.

Phytoestrogen class	Example of dietary source
Isoflavones	Legumes, lentils, chickpeas, soybean
Genistein	
Daidzein	
Glycitein	
Formononetin	
Biochanin A	
Coumestans	Young sprouting legumes
Coumestrol	e.g. clover and alfalfa sprouts
Lignans	Most cereals, linseed, fruit & vegetables
Matairesinol	
Secoisolariciresinol	
Lariciresinol	
Isolariciresinol	
Prenylated flavonoids	Some beers (Hops)
6-prenylnaringenin	
8-prenylnaringenin	
Xanthohumol	
Isoxanthohumol	

Fig. 5.4.1. Classes of phytoestrogens and common dietary sources.

Attachments

DNA Synthesis	genistein level	0	0,1	1	10	50	100
	-GW9662	100	88	78	68	36	28
	+GW9662	100	105	105	97	96	93
	-ICI182780	100	90	80	70	43	29
	+ICI182780	100	95	85	65	40	27
Collagen Synthesis	genistein level	0	0,1	1	10	50	100
	-GW9662	100	98	85	66	40	27
	+GW9662	100	100	99	97	96	95
	-ICI182780	100	97	81	65	35	26
	+ICI182780	100	97	81	65	36	27
Cell Number	genistein level	0	0,1	1	10	50	100
	-GW9662	100	86	76	64	28	25
	+GW9662	100	99,5	98,5	97	95	94
	-ICI182780	100	95	86	60	27	27
	+ICI182780	100	97	88	58	25	24

Table 1: DNA synthesis, Collagen synthesis and cell numbers of SMC cultures as percentage of control under the influence of different genistein levels ($\mu\text{ml/l}$); shwon with unblocked and blocked PPAR- γ receptor (-/+GW9662) and blocked and unblocked oestrogen receptor (-/+ICI182780)

Cell number						
Rosiglitazone	0	0,1	1	10	50	100
-GW9662	100	96	93	84	74	64
+GW9662	100	99	98	97,5	97	95

Table 2: Cell numbers of SMC cultures as percentage of control under the influence of different Rosiglitazone levels ($\mu\text{ml/l}$); shwon with unblocked and blocked PPAR- γ receptor (-/+GW9662)

Cell number						
	0	0,1	1	10	50	100
Rosiglitazone	100	96	90	80	70	60
Genistein	100	86	76	64	30	27

Table 3: Cell numbers of SMC cultures as percentage of control under the influence of different Rosiglitazone and Genistein levels ($\mu\text{ml/l}$)

Cell number	Control	Estradiol	Rosiglitazone	Genistein
-GW9662	100	53	75	62
+GW9662	100	55	97	90

Table 4: Cell numbers of SMC cultures as percentage of control under the influence of Estradiol, Rosiglitazone and Genistein ($\mu\text{ml/l}$)

DNA Synthesis	Control	Daidzein	Equol	Resveratrol	Genistein
-GW9662	100	60	80	70	40
+GW9662	100	86	80	84	88

Table 5: DNA synthesis of SMC cultures as percentage of control under the influence of Daidzein, Equol, Resveratrol and Genistein ($\mu\text{ml/l}$)

References

1. Thomas, B.F., et al., *Quantitative analysis of the principle soy isoflavones genistein, daidzein and glycitein, and their primary conjugated metabolites in human plasma and urine using reversed-phase high-performance liquid chromatography with ultraviolet detection.* J Chromatogr B Biomed Sci Appl, 2001. **760**(2): p. 191-205.
2. Griffith, A.P. and M.W. Collison, *Improved methods for the extraction and analysis of isoflavones from soy-containing foods and nutritional supplements by reversed-phase high-performance liquid chromatography and liquid chromatography-mass spectrometry.* J Chromatogr A, 2001. **913**(1-2): p. 397-413.
3. Nakamura, Y., S. Tsuji, and Y. Tonogai, *Determination of the levels of isoflavonoids in soybeans and soy-derived foods and estimation of isoflavonoids in the Japanese daily intake.* J AOAC Int, 2000. **83**(3): p. 635-50.
4. Liggins, J., R. Grimwood, and S.A. Bingham, *Extraction and quantification of lignan phytoestrogens in food and human samples.* Anal Biochem, 2000. **287**(1): p. 102-9.
5. Foster, W.G., et al., *Detection of phytoestrogens in samples of second trimester human amniotic fluid.* Toxicol Lett, 2002. **129**(3): p. 199-205.
6. Mazur, W., et al., *Isotope dilution gas chromatographic-mass spectrometric method for the determination of isoflavonoids, coumestrol, and lignans in food samples.* Anal Biochem, 1996. **233**(2): p. 169-80.
7. Doerge, D.R., et al., *Analysis of soy isoflavone conjugation in vitro and in human blood using liquid chromatography-mass spectrometry.* Drug Metab Dispos, 2000. **28**(3): p. 298-307.
8. Chang, H.C., et al., *Mass spectrometric determination of Genistein tissue distribution in diet-exposed Sprague-Dawley rats.* J Nutr, 2000. **130**(8): p. 1963-70.
9. Uehara, M., et al., *Rapid analysis of phytoestrogens in human urine by time-resolved fluoroimmunoassay.* J Steroid Biochem Mol Biol, 2000. **72**(5): p. 273-82.
10. Wang HJ, M.P., *Isoflavone Composition of American and Japanese Soybeans in Iowa: Effects of Variety, Crop Year and Location.* J Agric Food Chem., 1994. **42**(42): p. 1674-1677.
11. Liggins, J., et al., *Daidzein and genistein contents of vegetables.* Br J Nutr, 2000. **84**(5): p. 717-25.
12. Bingham, S.A., et al., *Phyto-oestrogens: where are we now?* Br J Nutr, 1998. **79**(5): p. 393-406.
13. Kim, J. and C. Kwon, *Estimated dietary isoflavone intake of Korean population based on National Nutrition Survey.* Nutr Res, 2001. **21**(7): p. 947-953.
14. Setchell, K.D., et al., *Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability.* Am J Clin Nutr, 2002. **76**(2): p. 447-53.
15. Day, A.J., et al., *Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity.* FEBS Lett, 1998. **436**(1): p. 71-5.
16. Allred, C.D., et al., *Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein.* Carcinogenesis, 2001. **22**(10): p. 1667-73.
17. Richelle, M., et al., *Hydrolysis of isoflavone glycosides to aglycones by beta-glucosidase does not alter plasma and urine isoflavone pharmacokinetics in postmenopausal women.* J Nutr, 2002. **132**(9): p. 2587-92.
18. Knight, D.C. and J.A. Eden, *A review of the clinical effects of phytoestrogens.* Obstet Gynecol, 1996. **87**(5 Pt 2): p. 897-904.
19. Sfakianos, J., et al., *Intestinal uptake and biliary excretion of the isoflavone genistein in rats.* J Nutr, 1997. **127**(7): p. 1260-8.

20. Coldham, N.G., et al., *Biotransformation of genistein in the rat: elucidation of metabolite structure by product ion mass fragmentology*. J Steroid Biochem Mol Biol, 1999. **70**(4-6): p. 169-84.
21. Adlercreutz, C.H., et al., *Soybean phytoestrogen intake and cancer risk*. J Nutr, 1995. **125**(3 Suppl): p. 757S-770S.
22. Watanabe, S., et al., *Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako)*. J Nutr, 1998. **128**(10): p. 1710-5.
23. Setchell, K.D., et al., *Comparing the pharmacokinetics of daidzein and genistein with the use of ^{13}C -labeled tracers in premenopausal women*. Am J Clin Nutr, 2003. **77**(2): p. 411-9.
24. Maskarinec, G., et al., *Dietary soy intake and urinary isoflavone excretion among women from a multiethnic population*. Cancer Epidemiol Biomarkers Prev, 1998. **7**(7): p. 613-9.
25. Bayer, T., T. Colnot, and W. Dekant, *Disposition and biotransformation of the estrogenic isoflavone daidzein in rats*. Toxicol Sci, 2001. **62**(2): p. 205-11.
26. Coldham, N.G. and M.J. Sauer, *Pharmacokinetics of [(14)C]Genistein in the rat: gender-related differences, potential mechanisms of biological action, and implications for human health*. Toxicol Appl Pharmacol, 2000. **164**(2): p. 206-15.
27. Clarke, R., et al., *Estrogens, phytoestrogens, and breast cancer*. Adv Exp Med Biol, 1996. **401**: p. 63-85.
28. Fitzpatrick, L.A., *Selective estrogen receptor modulators and phytoestrogens: new therapies for the postmenopausal women*. Mayo Clin Proc, 1999. **74**(6): p. 601-7.
29. Parker, M.G., *Structure and function of estrogen receptors*. Vitam Horm, 1995. **51**: p. 267-87.
30. Hall, J.M., J.F. Couse, and K.S. Korach, *The multifaceted mechanisms of estradiol and estrogen receptor signaling*. J Biol Chem, 2001. **276**(40): p. 36869-72.
31. Cowley, S.M., et al., *Estrogen receptors alpha and beta form heterodimers on DNA*. J Biol Chem, 1997. **272**(32): p. 19858-62.
32. Ogawa, S., et al., *The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro*. Biochem Biophys Res Commun, 1998. **243**(1): p. 122-6.
33. Pettersson, K., et al., *Mouse estrogen receptor beta forms estrogen response element-binding heterodimers with estrogen receptor alpha*. Mol Endocrinol, 1997. **11**(10): p. 1486-96.
34. Saunders, P.T., et al., *Differential expression of estrogen receptor-alpha and -beta and androgen receptor in the ovaries of marmosets and humans*. Biol Reprod, 2000. **63**(4): p. 1098-105.
35. Saunders, P.T., et al., *Differential expression of oestrogen receptor alpha and beta proteins in the testes and male reproductive system of human and non-human primates*. Mol Hum Reprod, 2001. **7**(3): p. 227-36.
36. Hawkins, M.B., et al., *Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts*. Proc Natl Acad Sci U S A, 2000. **97**(20): p. 10751-6.
37. Enmark, E., et al., *Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern*. J Clin Endocrinol Metab, 1997. **82**(12): p. 4258-65.
38. Reynolds, T., *New estrogen receptor adds complexity, recasts drug strategies*. J Natl Cancer Inst, 1999. **91**(17): p. 1445-7.

39. Inoue, S., et al., *An estrogen receptor beta isoform that lacks exon 5 has dominant negative activity on both ERalpha and ERbeta*. *Biochem Biophys Res Commun*, 2000. **279**(3): p. 814-9.
40. Ogawa, S., et al., *Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor of estrogen action in human*. *Nucleic Acids Res*, 1998. **26**(15): p. 3505-12.
41. Lemieux, P. and S. Fuqua, *The role of the estrogen receptor in tumor progression*. *J Steroid Biochem Mol Biol*, 1996. **56**(1-6 Spec No): p. 87-91.
42. Pfeffer, U., et al., *Alternative splicing of the estrogen receptor primary transcript normally occurs in estrogen receptor positive tissues and cell lines*. *J Steroid Biochem Mol Biol*, 1996. **56**(1-6 Spec No): p. 99-105.
43. Karas, R.H., et al., *Human vascular smooth muscle cells express an estrogen receptor isoform*. *FEBS Lett*, 1995. **377**(2): p. 103-8.
44. Brzozowski, A.M., et al., *Molecular basis of agonism and antagonism in the oestrogen receptor*. *Nature*, 1997. **389**(6652): p. 753-8.
45. Pike, A.C., et al., *Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist*. *Embo J*, 1999. **18**(17): p. 4608-18.
46. Fang, H., et al., *Structure-activity relationships for a large diverse set of natural, synthetic, and environmental estrogens*. *Chem Res Toxicol*, 2001. **14**(3): p. 280-94.
47. Blair, R.M., et al., *The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands*. *Toxicol Sci*, 2000. **54**(1): p. 138-53.
48. Kuiper, G.G., et al., *Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta*. *Endocrinology*, 1997. **138**(3): p. 863-70.
49. Milligan, S.R., et al., *The endocrine activities of 8-prenylnaringenin and related hop (*Humulus lupulus L.*) flavonoids*. *J Clin Endocrinol Metab*, 2000. **85**(12): p. 4912-5.
50. Zava, D.T. and G. Duwe, *Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro*. *Nutr Cancer*, 1997. **27**(1): p. 31-40.
51. Breinholt, V. and J.C. Larsen, *Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay*. *Chem Res Toxicol*, 1998. **11**(6): p. 622-9.
52. Paige, L.A., et al., *Estrogen receptor (ER) modulators each induce distinct conformational changes in ER alpha and ER beta*. *Proc Natl Acad Sci U S A*, 1999. **96**(7): p. 3999-4004.
53. Collins, B.M., J.A. McLachlan, and S.F. Arnold, *The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast*. *Steroids*, 1997. **62**(4): p. 365-72.
54. Mousavi, Y. and H. Adlercreutz, *Enterolactone and estradiol inhibit each other's proliferative effect on MCF-7 breast cancer cells in culture*. *J Steroid Biochem Mol Biol*, 1992. **41**(3-8): p. 615-9.
55. Jensen, E.V., *Steroid hormones, receptors, and antagonists*. *Ann N Y Acad Sci*, 1996. **784**: p. 1-17.
56. Beato, M., P. Herrlich, and G. Schutz, *Steroid hormone receptors: many actors in search of a plot*. *Cell*, 1995. **83**(6): p. 851-7.
57. Tsai, M.J. and B.W. O'Malley, *Molecular mechanisms of action of steroid/thyroid receptor superfamily members*. *Annu Rev Biochem*, 1994. **63**: p. 451-86.
58. Nagel, S.C., F.S. vom Saal, and W.V. Welshons, *The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays:*

- physiology of delivery modifies estrogenic activity.* Proc Soc Exp Biol Med, 1998. **217**(3): p. 300-9.
59. Anthony, M.S., et al., *Soybean isoflavones improve cardiovascular risk factors without affecting the reproductive system of peripubertal rhesus monkeys.* J Nutr, 1996. **126**(1): p. 43-50.
60. Awoniyi, C.A., et al., *Reproductive sequelae in female rats after in utero and neonatal exposure to the phytoestrogen genistein.* Fertil Steril, 1998. **70**(3): p. 440-7.
61. Fritz, W.A., et al., *Dietary genistein down-regulates androgen and estrogen receptor expression in the rat prostate.* Mol Cell Endocrinol, 2002. **186**(1): p. 89-99.
62. Medlock, K.L., W.S. Branham, and D.M. Sheehan, *The effects of phytoestrogens on neonatal rat uterine growth and development.* Proc Soc Exp Biol Med, 1995. **208**(3): p. 307-13.
63. Balmir, F., et al., *An extract of soy flour influences serum cholesterol and thyroid hormones in rats and hamsters.* J Nutr, 1996. **126**(12): p. 3046-53.
64. Divi, R.L. and D.R. Doerge, *Inhibition of thyroid peroxidase by dietary flavonoids.* Chem Res Toxicol, 1996. **9**(1): p. 16-23.
65. Mitsuma, T., et al., *The effects of Soybean Diet on Thyroid Hormone and Thyrotropin Levels in Aging Rats.* Endocr Regul, 1998. **32**(4): p. 183-186.
66. Lephart, E.D., et al., *Phytoestrogens decrease brain calcium-binding proteins but do not alter hypothalamic androgen metabolizing enzymes in adult male rats.* Brain Res, 2000. **859**(1): p. 123-31.
67. Atluru, S. and D. Atluru, *Evidence that genistein, a protein-tyrosine kinase inhibitor, inhibits CD28 monoclonal-antibody-stimulated human T cell proliferation.* Transplantation, 1991. **51**(2): p. 448-50.
68. Flynn, K.M., et al., *Effects of genistein exposure on sexually dimorphic behaviors in rats.* Toxicol Sci, 2000. **55**(2): p. 311-9.
69. Lephart, E.D., et al., *Neurobehavioral effects of dietary soy phytoestrogens.* Neurotoxicol Teratol, 2002. **24**(1): p. 5-16.
70. Patisaul, H.B., et al., *Soy isoflavone supplements antagonize reproductive behavior and estrogen receptor alpha- and beta-dependent gene expression in the brain.* Endocrinology, 2001. **142**(7): p. 2946-52.
71. Alekel, D.L., et al., *Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women.* Am J Clin Nutr, 2000. **72**(3): p. 844-52.
72. Alexandersen, P., et al., *Ipriflavone in the treatment of postmenopausal osteoporosis: a randomized controlled trial.* Jama, 2001. **285**(11): p. 1482-8.
73. Clifton-Bligh, P.B., et al., *The effect of isoflavones extracted from red clover (Rimostil) on lipid and bone metabolism.* Menopause, 2001. **8**(4): p. 259-65.
74. Felson, D.T., et al., *The effect of postmenopausal estrogen therapy on bone density in elderly women.* N Engl J Med, 1993. **329**(16): p. 1141-6.
75. Fitzpatrick, L.A., *Phytoestrogens--mechanism of action and effect on bone markers and bone mineral density.* Endocrinol Metab Clin North Am, 2003. **32**(1): p. 233-52, viii.
76. Humfrey, C.D., *Phytoestrogens and human health effects: weighing up the current evidence.* Nat Toxins, 1998. **6**(2): p. 51-9.
77. Nakajima, D., et al., *Suppressive effects of genistein dosage and resistance exercise on bone loss in ovariectomized rats.* J Physiol Anthropol Appl Human Sci, 2001. **20**(5): p. 285-91.
78. Arai, N., et al., *Estrogen receptor beta mRNA in colon cancer cells: growth effects of estrogen and genistein.* Biochem Biophys Res Commun, 2000. **270**(2): p. 425-31.

79. Gao, C.M., et al., *Protective effect of allium vegetables against both esophageal and stomach cancer: a simultaneous case-referent study of a high-epidemic area in Jiangsu Province, China*. Jpn J Cancer Res, 1999. **90**(6): p. 614-21.
80. Aronson, W.J., et al., *Decreased growth of human prostate LNCaP tumors in SCID mice fed a low-fat, soy protein diet with isoflavones*. Nutr Cancer, 1999. **35**(2): p. 130-6.
81. Alexandersen, P., et al., *Dietary phytoestrogens and estrogen inhibit experimental atherosclerosis*. Climacteric, 2001. **4**(2): p. 151-9.
82. van der Schouw, Y.T., et al., *Higher usual dietary intake of phytoestrogens is associated with lower aortic stiffness in postmenopausal women*. Arterioscler Thromb Vasc Biol, 2002. **22**(8): p. 1316-22.
83. Tobin, J.F. and L.P. Freedman, *Nuclear receptors as drug targets in metabolic diseases: new approaches to therapy*. Trends Endocrinol Metab, 2006. **17**(7): p. 284-90.
84. Mardikar, H.M. and D. Mukherjee, *Current endovascular treatment of peripheral arterial disease*. Prog Cardiovasc Nurs, 2007. **22**(1): p. 31-7.
85. Katz, E., et al., *[Sudden cardiac death: epidemiology and modern therapy]*. Rev Med Suisse, 2007. **3**(96): p. 302-7.
86. Kannel, W.B., *Incidence and epidemiology of heart failure*. Heart Fail Rev, 2000. **5**(2): p. 167-73.
87. Mannava, K. and S.R. Money, *Current management of peripheral arterial occlusive disease : a review of pharmacologic agents and other interventions*. Am J Cardiovasc Drugs, 2007. **7**(1): p. 59-66.
88. Safar, M.E. and H. Smulyan, *Atherosclerosis, arterial stiffness and antihypertensive drug therapy*. Adv Cardiol, 2007. **44**: p. 331-51.
89. Szmitko, P.E., et al., *Adiponectin and cardiovascular disease: state of the art?* Am J Physiol Heart Circ Physiol, 2007. **292**(4): p. H1655-63.
90. Van Gaal, L.F., I.L. Mertens, and C.E. De Block, *Mechanisms linking obesity with cardiovascular disease*. Nature, 2006. **444**(7121): p. 875-80.
91. Gornik, H.L. and M.A. Creager, *Contemporary management of peripheral arterial disease: I. Cardiovascular risk-factor modification*. Cleve Clin J Med, 2006. **73** Suppl 4: p. S30-7.
92. Kanter, J.E., et al., *Do glucose and lipids exert independent effects on atherosclerotic lesion initiation or progression to advanced plaques?* Circ Res, 2007. **100**(6): p. 769-81.
93. Ward, S., et al., *A systematic review and economic evaluation of statins for the prevention of coronary events*. Health Technol Assess, 2007. **11**(14): p. 1-178.
94. Roze, S., et al., *A health economic model to determine the long-term costs and clinical outcomes of raising low HDL-cholesterol in the prevention of coronary heart disease*. Curr Med Res Opin, 2006. **22**(12): p. 2549-56.
95. Dubey, R.K., et al., *Sex hormones and hypertension*. Cardiovasc Res, 2002. **53**(3): p. 688-708.
96. Rossouw, J.E., et al., *Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial*. Jama, 2002. **288**(3): p. 321-33.
97. Lobo, R.A., *Surgical menopause and cardiovascular risks*. Menopause, 2007. **14**(3 Suppl): p. 562-6.
98. Haney, A.F. and R.A. Wild, *Options for hormone therapy in women who have had a hysterectomy*. Menopause, 2007. **14**(3 Suppl): p. 592-7.

99. Dubey, R.K., et al., *Catecholamines block the antimitogenic effect of estradiol on human coronary artery smooth muscle cells*. J Clin Endocrinol Metab, 2004. **89**(8): p. 3922-31.
100. Dubey, R.K., et al., *Cytochromes 1A1/1B1- and catechol-O-methyltransferase-derived metabolites mediate estradiol-induced antimitogenesis in human cardiac fibroblast*. J Clin Endocrinol Metab, 2005. **90**(1): p. 247-55.
101. Krauss, R.M., *Individualized hormone-replacement therapy?* N Engl J Med, 2002. **346**(13): p. 1017-8.
102. Nguyen, L., et al., *Hormone replacement therapy and peripheral vascular disease in women*. Vasc Endovascular Surg, 2004. **38**(6): p. 547-56.
103. Dubey, R.K., et al., *Hormone replacement therapy and cardiovascular disease: what went wrong and where do we go from here?* Hypertension, 2004. **44**(6): p. 789-95.
104. Dubey, R.K., et al., *Vascular consequences of menopause and hormone therapy: importance of timing of treatment and type of estrogen*. Cardiovasc Res, 2005. **66**(2): p. 295-306.
105. Dubey, R.K. and E.K. Jackson, *Cardiovascular protective effects of 17 β -estradiol metabolites*. J Appl Physiol, 2001. **91**(4): p. 1868-83.
106. Dubey, R.K., S.P. Tofovic, and E.K. Jackson, *Cardiovascular pharmacology of estradiol metabolites*. J Pharmacol Exp Ther, 2004. **308**(2): p. 403-9.
107. Stampfer, M.J. and G.A. Colditz, *Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence*. Prev Med, 1991. **20**(1): p. 47-63.
108. Hulley, S., et al., *Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women*. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. Jama, 1998. **280**(7): p. 605-13.
109. Viscoli, C.M., et al., *A clinical trial of estrogen-replacement therapy after ischemic stroke*. N Engl J Med, 2001. **345**(17): p. 1243-9.
110. Dubey, R.K., et al., *Tibolone and its metabolites induce antimitogenesis in human coronary artery smooth muscle cells: role of estrogen, progesterone, and androgen receptors*. J Clin Endocrinol Metab, 2004. **89**(2): p. 852-9.
111. Anthony, M.S., et al., *Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys*. Arterioscler Thromb Vasc Biol, 1997. **17**(11): p. 2524-31.
112. Cassidy, A., et al., *Critical review of health effects of soyabean phyto-oestrogens in post-menopausal women*. Proc Nutr Soc, 2006. **65**(1): p. 76-92.
113. Hall, W.L., et al., *Soy-isoflavone-enriched foods and inflammatory biomarkers of cardiovascular disease risk in postmenopausal women: interactions with genotype and equol production*. Am J Clin Nutr, 2005. **82**(6): p. 1260-8; quiz 1365-6.
114. Hasler, C.M., *The cardiovascular effects of soy products*. J Cardiovasc Nurs, 2002. **16**(4): p. 50-63; quiz 75-6.
115. Hermansen, K., et al., *Beneficial effects of a soy-based dietary supplement on lipid levels and cardiovascular risk markers in type 2 diabetic subjects*. Diabetes Care, 2001. **24**(2): p. 228-33.
116. Wagner, J.D., et al., *Dietary soy protein and estrogen replacement therapy improve cardiovascular risk factors and decrease aortic cholesteryl ester content in ovariectomized cynomolgus monkeys*. Metabolism, 1997. **46**(6): p. 698-705.
117. Honore, E.K., et al., *Soy isoflavones enhance coronary vascular reactivity in atherosclerotic female macaques*. Fertil Steril, 1997. **67**(1): p. 148-54.

118. Kurowska, E.M., et al., *Effects of substituting dietary soybean protein and oil for milk protein and fat in subjects with hypercholesterolemia*. Clin Invest Med, 1997. **20**(3): p. 162-70.
119. Mackey, R., A. Ekangaki, and J.A. Eden, *The effects of soy protein in women and men with elevated plasma lipids*. Biofactors, 2000. **12**(1-4): p. 251-7.

Acknowledgements

First I like to thank Dr. Dubey for making this interesting subject available for my dissertation and for his support throughout the whole work. It was a pleasure working with him.

Furthermore I want to thank my parents for always supporting me during my studies and this dissertation.

Curriculum vitae

–

Eva-Maria Boogen

PERSÖNLICHE ANGABEN

Name: Eva-Maria Boogen
Anschrift: Mielenforster Kirchweg 36, D-51069 Köln
Geburtsdatum / -ort: 09.11.1973 in Offenbach
Staatsangehörigkeit: Deutsch

SCHULAUSBILDUNG IN- UND AUSLAND

1980 – 1984 Grundschule Bollenberg in Haan
1984 – 1990 Städt. Gymnasium in Haan
1990 – 1991 Southern Lehigh High School in Center Valley, USA
Amerikanischer High-School Abschluss „Graduation“
1991 – 05/1993 Oberstufe und Abitur am Städt.Gymnasium in Haan

BERUFSBILDUNG

09/1993 – 09/1996 Ausbildung zur Bankkauffrau und Berufstätigkeit in der Stadt-Sparkasse Haan

STUDIUM & AUSBILDUNG

10/1996 – 04/2003 Studium der Humanmedizin an der Heinrich-Heine Universität Düsseldorf
Physikum 08/1999, 1. Staatsexamen 03/2001, 2. Staatsexamen 08/2002
04/2003 – 04/2004 Studium der Humanmedizin an der Ludwig-Maximilians-Universität München
3. Staatsexamen am 20.04.2004
08/2004 – 07/2007 Assistenzärztin, Ausbildung zur Fachärztin für Gynäkologie und Geburtshilfe, Spital Zollikerberg
seit 08/2007 Assistenzärztin, Ausbildung zur Fachärztin für Gynäkologie und Geburtshilfe, St. Marien-Hospital Bonn