DIRECT AND INDIRECT ENDOCRINE DISRUPTION

Aromatase and estrogen receptor-mediated processes in breast cancer development

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DIRECTE EN INDIRECTE HORMOONVERSTORING

Aromatase en oestrogeenreceptor gemedieerde processen

in de ontwikkeling van borstkanker

(met een samenvatting in het Nederlands)

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Marjoke Heneweer

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promotor: Prof. Dr. M. van den Berg,
Institute for Risk Assessment Sciences, Universiteit Utrecht.

co-promotor: Dr. J.T. Sanderson,
Institute for Risk Assessment Sciences, Universiteit Utrecht.

DIRECT AND INDIRECT ENDOCRINE DISRUPTION

Aromatase and estrogen receptor-mediated processes in breast cancer development

Marjoke Heneweer

Institute for Risk Assessment Sciences (IRAS)
Utrecht University
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The studies described in this thesis were carried out at the Institute for Risk Assessment Sciences, Utrecht University, PO Box 80176, 3508 TD Utrecht, The Netherlands.

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I love deadlines. I like the whooshing sound they make as they fly by

Douglas Adams (1952-2001)

Contents

List of abbreviations

17β-HSD	17β-hydroxysteroid dehydrogenase
2-OHE	2-hydroxy estrogen
4-MBC	3-(4-methylbenzylidene) camphor
4-OHE	4-hydroxy estrogen
8-Br-cAMP	8-bromo-cyclic adenosine monophosphate
AR	androgen receptor
ATR	atrazine
bp	basepair
BP-1	2,4-dihydroxybenzophenone, benzophenone-1
BP-3	2-hydroxy-4-methoxybenzophenone, benzophenone-3
BP-4	4-hydroxybenzophenone
BPA	bisphenol-A
C50	concentration at wich a compound causes a 50% increase in response
cAMP	cyclic adenosine monophosphate
CAR	carbendazim
CE	catechol estrogen
$CHCl_3$	chloroform
COMT	catechol-*O*-methyl transferase
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
CYP19	cytochrome P450 19, aromatase
CYP1A1	cytochrome P450 1A1
CYP1A2	cytochrome P450 1A2
CYP1B1	cytochrome P450 1B1
CYP3A4	cytochrome P450 3A4
CYP3A5	cytochrome P450 3A5
DCC	dextran-coated charcoal
DDT	dichlorodiphenyltrichloroethane
DES	diethylstilbestrol
DEX	dexamethasone
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
E1	estrone
E2	17β-estradiol
EC_{50}	concentration at which compound elicits 50% of its maximum effect
EDC	endocrine-disrupting chemical
EEQ	estrogen equivalents
ELISA	enzyme-linked immunosorbent assay
EPO	epoxyconazole
ER	estrogen receptor
EtBr	ethidium bromide

EtOH	ethanol
FAD	fadrozole
FCS	fetal calf serum
GAS	interferon gamma activation site
GR	glucocorticoid receptor
H295R	human adrenocortical carcinoma cell line
IL-6	interleukin-6
IL-6sR	interleukin-6 soluble receptor
IMI	imidacloprid
Jak	janus kinase
kb	kilobase
K_i	inhibitor dissociation constant for enzyme
K_i'	inhibitor dissociation constant for enzyme-substrate complex
K_M	concentration of substrate that leads to half V_{max}
LMA	low melting agarose
MCF-7	human mammary carcinoma cell line
MeOH	methanol
$MeSO_2$-PCB	methylsulfonyl polychlorinated biphenyl
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NMA	normal melting agarose
OMC	octyl methoxy cinnamate
o-p'-DDE	*ortho-para*-dichlorodiphenyldichloroethylene
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PGE2	prostaglandin E2
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMS	premenstrual syndrome
p-p'-DDE	*para-para*-dichlorodiphenyldichloroethylene
PRO	prochloraz
RP	relative potency
rt-PCR	reverse transcriptase polymerase chain reaction
TNF-α	tumor necrosis factor-α
U	units
UTR	untranslated region
UV	ultraviolet

General introduction 1

The endocrine system

The endocrine system is a complex regulatory system that maintains homeostasis in the body. It consists of many organs, which produce a number of different hormones. These hormones are directly released into the bloodstream by endocrine glands. Subsequently, they act on target cells that are located at different sites in the body and which contain specific hormone-binding receptors either on their membrane or in the cytosol. Once a hormone has entered the cell and interacted with its receptor it can exert its action by stimulation of second messenger pathways or by entering the nucleus and enhancing or decreasing gene transcription of specific hormone-responsive genes. There are different types of hormones. One can differentiate between proteins, catecholamines, and steroids.[1]

Proteins bind to a receptor on the cell membrane of, for example thyroid cells, and stimulate the adenylate cyclase enzyme, which leads to enhanced cyclic adenosine monophospate (cAMP) production. The production of this second messenger can in turn lead to hormone synthesis within the target cells.

Catecholamines are secreted by the adrenal gland. The adrenal gland consists of two regions, the medulla and the cortex that are completely different in form and function. Catecholamines are mainly produced in the medulla of the adrenal gland. Examples of catecholamines are epinephrine and norepinephrine. These hormones play a role in the so called ‘fright, flight or fight’-reactions. They increase blood pressure, heart rate, glucose level and oxidative metabolism. Furthermore, they decrease peristaltic movements of the intestines. Large quantities of these hormones can be secreted quickly from granules in case of an emergency situation.

Steroid hormones are secreted by the gonads and the cortex of the adrenal glands, which consists of three layers. Cells in the cortex contain many fat droplets that can hold the steroid hormone precursor cholesterol. The outer layer, the zona glomerulosa, produces the mineralocorticoids, which are responsible for increasing sodium and decreasing potassium concentrations in the blood resulting in fluid retention. The zonae fasciculata and reticularis (innermost zone) produce the glucocorticoids and sex steroids, respectively. Glucocorticoids are mainly responsible for protein, lipid and carbohydrate metabolism. Furthermore, they elicit a suppressive

effect on the immune system. Quantitatively, sex steroid production in the adrenal cortex only minimally contributes to the total sex steroid production in comparison with production in the gonads.

Testes consist of intertwined seminiferous tubules between which Leydig cells and Sertoli cells are located. Within the tubules, the first steps of spermatozoa production takes place and Sertoli cells are responsible for further development and maturation of the spermatozoa. In addition to production of gametes, gonads produce sex hormones. Leydig cells produce testosterone that is released into the bloodstream.

Ovaries consist of follicles embedded in stromal cells. Furthermore, follicles consist of an unfertilized egg surrounded by thecal and granulosa cells. Thecal cells are capable of synthesizing androgens, which are precursors of estrogens. In turn, granulosa cells convert androgens to estrogens using the aromatase enzyme.

Steroid hormones pass through the cell membrane of their target cells and bind to specific receptors located within the cytoplasm or on the nuclear membrane. After dimerization, the receptor undergoes conformational changes, making it competent to bind to hormone-responsive elements on the DNA. The hormone-receptor complexes are translocated to the nucleus and bind to DNA, where they act as transcription factors. Transcription factors have the ability to either stimulate or inhibit gene transcription and subsequent protein synthesis. This whole process can take several hours in contrast to the action of protein hormones, which is often instantaneous.

Estrogens

Estrogens are steroid hormones that are important for growth, development and functioning of various target organs such as uterus, ovaries, vagina, mammary gland, testes and prostate gland. Furthermore, they play an important role in (sexual) behaviour. Estrogens move through the body via the bloodstream and enter various cells, but are retained in target cells by high affinity binding to estrogen receptors (ERs).[2] The human ER exists as at least 2 subtypes ERα and ERβ, which differ in the C-terminal ligand-binding domain, as well as in the N-terminal transactivation

domain.[3] Both subtypes differ in function, in tissue-distribution and ligand-binding affinities for estradiol and estrone. The latter observation could account for the differences in effects in different tissues after exposure to endogenous estrogens or exogenous compounds with estrogenic properties. Activation of the ER regulates transcription of specific genes leading to physiological responses. Activation of gene transcription takes up to several hours. However, estrogens can also elicit rapid, non-genomic effects, such as interference with second messenger pathways.

Estrogen biosynthesis and metabolism

In premenopausal women, the main estrogen-producing cells are the granulosa cells in the ovary. At that time, peripheral tissues, such as fat, adipose stromal cells and bone play a minor role in estrogen synthesis. However, after menopause, when gonadal estrogen synthesis ceases, these peripheral tissues become important sites of estrogen production. Estrogen biosynthesis starts with the precursor cholesterol. Via several enzymatic conversions the androgens androstenedione and testosterone are formed. In their turn, these androgens are direct precursors for estrone (E1) and 17β-estradiol (E2), respectively. The rate-limiting enzyme aromatase is able to convert androgens into estrogens. E1 can be converted to E2 and vice versa by two isoforms of 17β-hydroxysteroid dehydrogenase (17β-HSD).

Estrogens are metabolized into 2-hydroxycatechol estrogens by cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1A2 (CYP1A2), or into 4-hydroxycatechol estrogens by cytochrome P450 1B1 (CYP1B1). Also 16α-hydroxy estrogens are formed by cytochrome P450 3A4 and 3A5. The catechol estrogens may be detoxified by catechol-*O*-methyl transferase (COMT) [4-7] (figure 1).

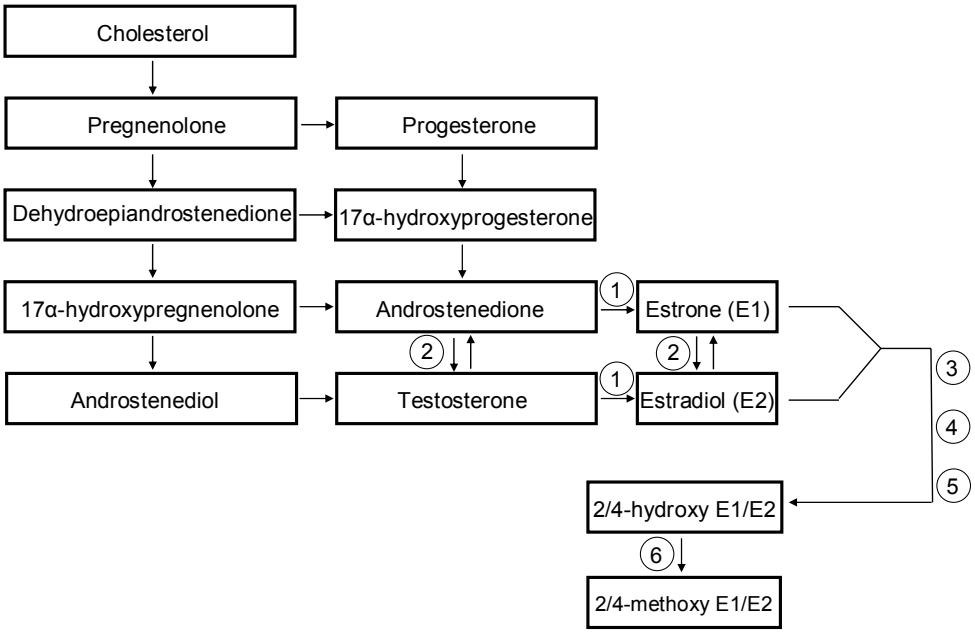

Figure 1: Estrogen biosynthesis and metabolism. The main enzymes are (1) aromatase, (2) 17β-hydroxysteroid dehydrogenase, (3) cytochrome P450 1A1, (4) cytochrome P450 1A2, (5) cytochrome P450 1B1 and (6) catechol-*O*-methyl transferase.

Aromatase

Aromatase (CYP19) is bound to the membrane of the endoplasmatic reticulum and comprises the ubiquitous flavoprotein, NADPH-cytochrome P450 reductase, and a unique cytochrome P450 (aromatase), which is exclusively expressed in estrogen producing cells.[8] Aromatase converts androgens into estrogens by aromatizing the A-ring of the androgen, using 3 molecules of molecular oxygen and 3 molecules NADPH per molecule of androgen (figure 2).[9-11]

In most vertebrates, aromatase has been detected in gonads and brain. In humans, aromatase is also present in placenta, adipose stromal cells, bone and various fetal tissues.[8] The aromatase protein is highly conserved with 50-90% amino

acid sequence identity among the species studied so far, including fish, birds, and mammals. However, there are significant differences in gene size (~2.6 kb in Medaka and ~34 kb in humans).[8] CYP19 is a single copy gene in humans, unlike in some other vertebrates.

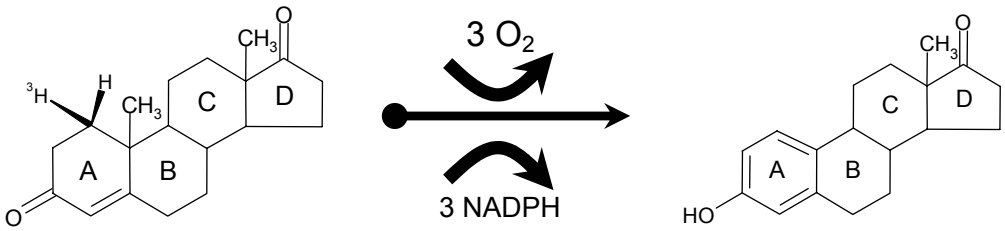

Figure 2: Aromatase uses 3 molecular oxygen molecules and 3 NADPH molecules to convert 1 androgen (C19) molecule into 1 estrogen molecule (C18).

Regulation of aromatase activity in human breast tissue

The human aromatase gene was mapped to the 15q21.1 chromosome.[12] Expression of the aromatase gene is regulated in a species- and tissue-specific manner (reviewed in [9,13]). In humans, 10 exons have been identified. Exons II-X encode the aromatase protein and 3'- untranslated region of the mRNA. The 5'-untranslated region contains several first exons that lie downstream from tissue-specific promoters.[14,15] Those promoters in turn are linked to different signal transduction pathways [8] (figure 3). The physiological environment of the tissue determines the main signal transduction pathway that is active, and subsequently, first exons are alternatively spliced onto a common site (GACT) just upstream from the translation initiation codon ATG in exon II. Although CYP19 expression is driven by tissue-specific promoters, the aromatase protein encoded in each of these tissues is identical.[8]

In healthy breast tissue, aromatase is mainly located in adipose stromal cells (fibroblasts) and aromatase gene transcription is mainly regulated by a glucocorticoids-dependent pathway, using promoter region I.4.[16,17] Class I cytokines,

such as TNF-α [18] and IL-6 [19,20] can stimulate the Jak1/STAT3 signalling pathway, which activates the interferon γ activation site (GAS) and hence increases aromatase gene expression via promoter region I.4.[21] For stimulation of aromatase gene transcription via class I cytokines, glucocorticoids and transcription factor Sp 1 are required.[20,22]

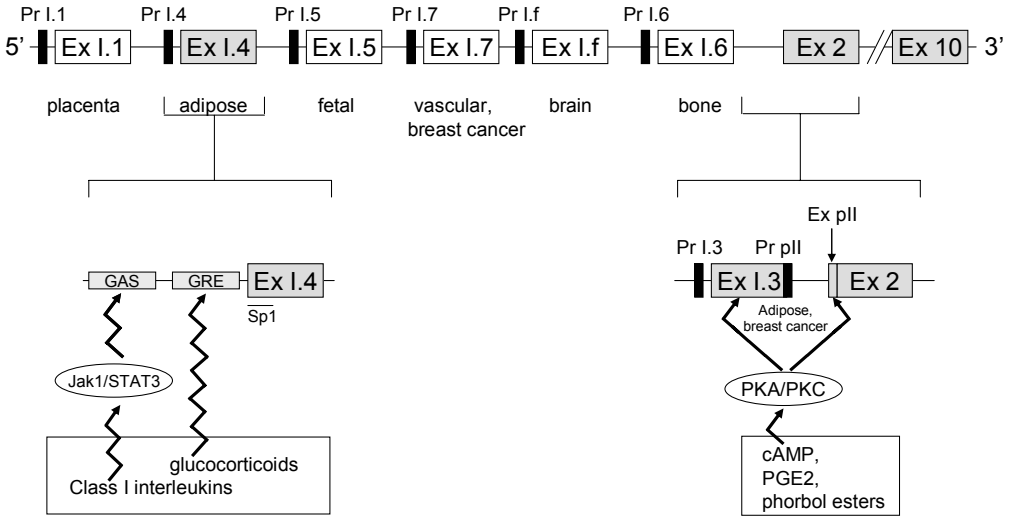

Figure 3: Aromatase promoter region. Transcription of tissue-specific exons is driven by promoters that are located upstream from each exon. In the human breast, the main first exons are I.4, I.3 and pII. Transcription of exon I.4 is activated by simultaneous binding of class I cytokines, glucocorticoids and Sp1 to its promoter region. Expression of exons I.3 and pII is stimulated by cAMP, PGE2 and phorbol esters via PKA and PKC. Coding region for aromatase is located in exons 2-10.

In breast tumors, it has been found that CYP19 transcripts derived from promoter regions I.3 and pII become more prevalent. These promoter regions are stimulated by cAMP through the protein kinase A (PKA) and by phorbol esters through the protein kinase C (PKC) phosphorylation pathways. Prostaglandin E2

(PGE2) is a major product secreted by breast tumor epithelial cells [23] and thought to be the most important stimulant of the I.3/pII promoters [24] by stimulating both PKA and PKC phosphorylation pathways. PGE2 interacts with two receptor subtypes in fibroblasts, EP_1 and EP_2. Upon binding of PGE2 to EP_1, the associated G-protein stimulates phospholipase C, resulting in formation of second messengers and activation of PKC. Subsequently, a cascade of serine/threonine phosphorylation events is initiated.[23] Upon binding of PGE2 to EP_2, the associated G-protein stimulates adenylate cyclase activity, resulting in elevated production of cAMP. Subsequently, the cAMP-responsive element (CRE) in promoter regions I.3 and pII binds CRE binding protein (CREB) which is phosphorylated via PKA-mediated pathways.[25] As a consequence, the stimulation elicited by PGE2 is equivalent to the combined responses of forskolin (enhancer of cAMP production, which activates PKA) and phorbol esters (which are activators of PKC).[24]

Estrogen hydroxylation and -methylation

A-ring hydroxylation of estrogens is mainly catalyzed by CYP1A1, CYP1A2 and CYP1B1. CYP1A1 and CYP1A2 are located in extra-hepatic and hepatic tissue, respectively and are responsible for the formation of 2-hydroxy estrogens (2-OHE). 4-Hydroxy estrogens (4-OHE) are formed by CYP1B1, which is mainly located in extra-hepatic tissues.[26-28] These catechol estrogens (CEs) are further metabolized by COMT, which methylates the molecules.[29,30] However, in the absence of COMT or if COMT activity/expression is compromised, oxidation of the CEs may occur leading to formation of quinones that are able to react with macromolecules in the cell. Quinones of 2-OHE can form stable DNA adducts that stay in the DNA unless repaired. Quinones of 4-OHE can form depurinating DNA adducts, which are potential initiators of carcinogenesis.[31,32]

17β-HSD

At least seven types of human 17β-HSD interconvert 17-ketosteroids (estrone) and 17-hydroxysteroids (17β-estradiol). The 17-hydroxy steroids are biologically more active than the ketones. The conversion of E1 to E2, is catalyzed by 17β-HSD type 1 and 7, using NADPH as cofactor. The conversion of E2 to E1, is

catalyzed by 17β-HSD type 2 which uses NAD+ as a cofactor. The enzymes are present in ovarian granulosa cells and other estrogen target tissues.[33]

Estrogen sulfatase and sulfotransferase

The addition of a sulfate group to estrogens is catalyzed by estrogen sulfotransferase type I and type II. Estrogen sulfates (ES) have a longer plasma half-life than E1 and E2 and have no estrogenic effects. Breast tumors of postmenopausal patients contain estrogen sulfates in equal,[34,35] or higher amounts than the unconjugated estrogens.[36] ES therefore act as a pool of estrogens in tissues and plasma.[37] Mobilization of the estrogenic E1 and E2 from ES is catalyzed by estrogen sulfatase.[35]

Contradictory results are described on the relative importance of sulfatase in estrogen production and breast cancer development. Sulfatase activity appears to be higher than aromatase activity in the same tissue type.[37] However, only 24% of the intratumoral estrogen was produced by the sulfatase enzyme.[38] Since estrogen sulfate formation is dependent on estrogen formation by aromatase, aromatase can be considered the most important enzyme in the regulation of estrogen synthesis.

Endocrine disruption

The World Health Organization defines an endocrine disrupting chemical (EDC) as "*an exogenous substance or mixture that alters function(s) of the endocrine system and causes adverse health effects in an intact organism, or its progeny, or (sub)populations*".[39] Both synthetic chemicals, such as polychlorinated biphenyls (PCBs) and naturally occurring chemicals, such as phytochemicals can disrupt the endocrine system.

There are multiple mechanisms through which EDCs can exert their effect. EDCs can, for example, bind directly to a steroid hormone receptor and subsequently block (antagonism) or induce (agonism) the response. If they do not bind to a steroid hormone receptor, EDCs may alternately interfere with the cascade of responses in the cell that are elicited by hormone-receptor activation. Also the feedback systems

that regulate hormone synthesis may be disturbed by EDCs. This can lead to induced or reduced hormone synthesis, with subsequently elevated or decreased levels of steroid hormones, possibly resulting in adverse effects on reproduction, development and/or hormone-responsive tumor growth.

Direct estrogenic effects

Estrogenic effects of chemicals have been subject of extensive research during the past decades. Classical studies on estrogen receptor-binding have been used to study the estrogenic effects of new chemicals and environmental contaminants.

For example, bisphenol-A (BPA) that is used primarily for production of polycarbonate plastic and epoxy resins and for food and beverage containers, binds to the ER. Although exposure to BPA leads to both ERα and ERβ- mediated gene transcription, BPA seems to prefer ERβ over ERα in receptor-binding experiments. Relative potency of BPA in reporter gene experiments was 4 orders of magnitude less than that of E2.[40] Phytochemicals with estrogenic properties, including genistein, coumestrol and zearalenone are capable of stimulating transcriptional activity of both ER subtypes.[3] In a rapid yeast estrogen bioassays stably expressing human ERα and ERβ, relative potencies of these phytochemicals were 2 to 3 orders of magnitude lower than that of E2.[41]

A third example of a widely studied environmental contaminant is DDT. DDT is an insecticide that was first used during world war II for control of lice and mosquitoes that were responsible for the spread of typhoid fever and malaria, respectively.[42] In 1972, DDT was banned by the U.S. Environmental Protection Agency after it had been used extensively as an insecticide in forestry and agriculture. At the peak of DDT production in the early 1960s, 81 million kg was produced per year.[43]

Endocrine disruptive effects of DDT include reproductive malformations, feminization and eggshell thinning in avian species.[44] Also sex reversal in Medaka fish [45] and changes in sexual differentiation and behavior in mice [46] have been observed. Breakdown products of DDT, such as *o-p'*-DDE and *p-p'*-DDE, have been

identified as environmental estrogens.[47] They support the growth of estrogen-dependent breast tumors in rats and are stored in human adipose tissue resulting in levels increasing with age. However, since the ban on DDT production in 1972, levels have been declining.[48]

Interactions with the ER could possibly lead to adverse effects in the human body and therefore, in the US, companies are obliged to test their compounds for estrogenic effects before being allowed to bring the product onto the market. In Japan and Europe, legislation forcing companies to perform tests for estrogenicity, is being introduced.[39]

Until now, estrogenic effects of compounds have mainly been investigated using *in vitro* models that include cell proliferation studies, androgen receptor- and ER-binding, steroidogenic enzyme activity or hormone synthesis, and (reporter) gene assays using transient or stably transfected cells.[49] A major backdraw of *in vitro* studies is that results have to be extrapolated to the *in vivo* situation. *In vitro* studies often require isolation of cells that normally are part of a functioning organ system. Paracrine interactions between different cell types do not occur in the *in vitro* situation, which may lead to observations that are not relevant for the *in vivo* situation. Furthermore, in *in vitro* studies one can choose the concentration to which the cells are exposed. However, in *in vivo* experiments it is more difficult to tell how much of the test compound reaches the target cells and prediction of *in vivo* effects is complicated. Therefore, *in vivo* effects often have to be assessed using animal studies that look at reproductive organ weights and histology, serum hormone levels, gene activation, protein synthesis, behavior, growth, development, pregnancy maintenance, and/or anatomy/morphology after exposure of the animal to the compounds.

Indirect estrogenic effects

Recently, studies on chemicals affecting estrogen biosynthesis have attracted increasing attention. By enhancing estrogen synthesis, chemicals may exert an indirect estrogenic effect. For example, the widely used herbicide atrazine

does not bind to the ER but is capable of increasing aromatase activity approximately 3-fold in the human adrenocorticocarcinoma cell line H295R,[50,51] possibly leading to an increase in estrogen biosynthesis.

Several xenobiotics have been shown to affect aromatase enzyme activity. Various imidazole-like fungicides, such as imazalil and prochloraz are aromatase inhibitors. At the same time, vinclozolin and atrazine are capable of elevating aromatase enzyme activity through increased gene transcription.[51,52] Diindolylmethane, which is a dimeric condensation product of indole-3-carbinol, a constituent of cruciferous vegetables, was also shown to induce aromatase activity about 2-fold.[53]

Implications of changes in aromatase activity

Changes in aromatase activity may lead to increased or decreased estrogen production and subsequently increased or decreased local or systemic estrogen levels in the body.

Decreased estrogen levels may in turn lead to severe depression in women who suffer from pre-menstrual syndrome (PMS) or from postnatal depression.[54] Lower levels of estrogens can also lead to impaired fertility, as estrogens play a pivotal role in regulating the menstrual cycle in women in the reproductive age. Bone demineralization and osteoporosis is also a common effect of decreased estrogen levels in post-menopausal women.[55]

In contrast, elevated estrogen levels can for example, lead to a cardioprotective effect.[56] Also, symptoms of menopause are often treated with estrogen supplementation. However, elevated estrogen levels are associated with increased risk of breast cancer. Estrogens can stimulate hormone-responsive breast tumor growth and estrogen metabolism. Estrogen-metabolism may result in the production of genotoxic metabolites that are capable of initiating DNA damage.[32,57]

Besides ER-antagonists such as tamoxifen and raloxifen, aromatase inhibitors are frequently used as therapy for postmenopausal breast cancer.[58] By blocking aromatase activity, estrogen production is inhibited and hormone-dependent

tumors are deprived from estrogens resulting in growth arrest or even tumor regression.

Breast cancer development

Malignant tumors of the mammary gland epithelium are the most common type of cancer in women. In the year 2000, 373,000 women died of breast cancer more than 1,000,000 new cases were diagnosed worldwide.[59,60] Approximately 60% of all breast tumors are estrogen-responsive and depend on estrogens for growth.[61,62] Therefore, chemicals that show estrogenic properties, might be able to interfere with breast tumor growth. Epithelial breast tumor cells express ERs on their cell membrane to which endogenous and/or exogenous estrogens can bind. This interaction of estrogenic chemicals with the ER may result in activation or blocking of the ER with subsequent increased or decreased estrogenic effect in the cells.

Although ovarian estrogens are involved in the onset of breast cancer, more than 60% of new breast cancer cases are diagnosed after menopause, indicating a variable lag period between tumor initiation and diagnosis. After menopause estrogen production ceases to 10% of pre-menopausal levels.[34] Tissue concentrations of estrogens, however, do not differ significantly between pre- and postmenopausal patients.[63] This contradiction suggests that postmenopausal breast tumors depend on local estrogen synthesis, rather than circulating estrogens.

Cell-culture and nude mouse experiments using aromatase-transfected MCF-7 cells demonstrate that aromatase expressed in breast cancer cells play a role in stimulating the growth of tumors in an autocrine manner. However, breast cancer epithelial cells are embedded in adipose tissue and breast tumors can consist for 50% of adipose stromal cells.[64] In these tumors, predominantly stromal spindle cells and not malignant epithelial cells stained positive for aromatase using a polyclonal antibody,[64] suggesting estrogens are formed in the surrounding stromal cells. Furthermore, co-culture experiments using aromatase-transfected cells together with untransfected cells, showed that estrogens produced by the transfected cells could also stimulate the growth of untransfected cells in a paracrine manner.[65]

In case of an estrogen-responsive breast tumor, the E2 that is secreted by surrounding fibroblasts enhances tumor epithelial cell growth. In turn, increased numbers of epithelial tumor cells, secrete increased amounts of factors, which when diffused to fibroblasts, can induce aromatase gene expression in those cells. This results in the completion of a positive feedback loop that is capable of accelerating estrogen-responsive tumor growth, and forms the basis for a breast tumor promotion model.

Reed *et al* [66] formulated a hypothesis on a mechanism of regulation of aromatase activity and estrogen-dependent breast tumor growth. Breast tumor tissue consists of malignant epithelial cells, fibroblasts, adipose cells, macrophages and lymphocytes. Macrophages and lymphocytes secrete IL-6, IL-6 soluble receptor (IL-6sR) and TNF-α. In addition, fibroblasts secrete IL-6 and adipocytes secrete TNF-α. IL-6 is able to induce aromatase activity in fibroblasts. The effect of IL-6 is markedly potentiated by its soluble receptor, which is secreted by malignant epithelial cells upon estradiol stimulation. Complex formation of IL-6 with either the membrane bound or soluble form of its receptor induces homodimerization of the ubiquitously expressed signal-transducer gp130 into a hexameric complex, leading to activation of intracellular signaling cascades.[67] TNF-α induces aromatase activity through stimulation of IL-6sR release.[68]

PGE2 is a major secretory product of breast tumor epithelial cells [23] and an important factor in the regulation of IL-6 production in fibroblasts [69] and PGE2 stimulation of aromatase activity was enhanced by addition of IL-6sR.[70] These observations suggest that PGE2 stimulates aromatase activity partly through stimulation of IL-6 production. Furthermore, PGE2 is a potent stimulator of the PKA and PKC second messenger pathways and via this mechanism it can induce aromatase gene transcription through the pII and I.3 promoter regions in breast cancer tissue.[24]

Direct and indirect estrogenic effects

To assess effects of chemicals on breast cancer development, an *in vitro* breast cancer model containing pathways for both direct (acting on the ER) and

indirect (aromatase induction) estrogenicity would be an appropriate tool. *In vitro* assays have mainly been performed in systems consisting of only one cell type. Potentiation of estrogenic effects through paracrine interactions between epithelial and stromal cells, as described earlier, is not accounted for in these test systems. A co-culture system of MCF-7 cells and fibroblasts would be a useful addition to the battery of *in vitro* screening methods for estrogenic effects, as it would be more realistic. Such a co-culture system would allow for detection of chemicals that can interfere with the aromatase enzyme, as well as the ER in one interactive system.

Scope and objectives of this thesis

The general hypothesis of this thesis is that (anti)-estrogenic effects can be elicited by compounds that bind to the ER, as well as by compounds that induce or inhibit aromatase gene transcription and/or enzyme activity. Furthermore, we have assumed that a positive feedback loop exists between estrogen-responsive breast tumor epithelial cells and surrounding stromal cells, supporting breast tumor growth and development.

Studies in this thesis describe effects of several classes of xenobiotics on MCF-7 cell proliferation and pS2 gene transcription. Furthermore, effects on aromatase enzyme activity and gene transcription have been studied *in vitro*. Another goal of this thesis is to develop an *in vitro* breast cancer model to account for paracrine interactions between tumor epithelial cells and surrounding adipose tissue that are capable of establishing a positive feedback loop.

Chapter 2, describes the effects of commercially available UV-filters on estrogen-mediated pS2 expression in MCF-7 cells. UV-filters have been tested individually but also in binary and multi-component mixtures to test for additivity.

In **chapter 3**, the use of the human adrenocortical carcinoma cell line H295R and the rat Leydig cell R2C cell line as tools to study aromatase activity has been assessed. Several currently used herbicides and fungicides have been tested for their ability to alter aromatase activity in both cell lines.

Chapter 4 describes the effects of ubiquitously present methyl sulfonyl polychlorinated biphenyl ($MeSO_2$-PCB) metabolites on promoter-specific aromatase gene expression and aromatase activity in primary human mammary fibroblasts.

The development and validation of an *in vitro* breast cancer model is described in **chapter 5.** Co-cultures of MCF-7 and primary human fibroblasts have been used to describe direct and indirect effects of a known aromatase modulator, the synthetic glucocorticoid dexamethasone (DEX) and the known estrogens estradiol (E2), diethylstilbestrol (DES) and bisphenol A (BPA), and the synthetic anti-estrogen ICI 182,780 on pS2 gene transcription in MCF-7 cells and aromatase gene transcription in fibroblasts.

Chapter 6 uses co-culture experiments to describe estrogenic effects of UV-filter metabolites and **chapter 7** comprises a general discussion of results obtained in the previous chapters.

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Additive estrogenic effects of mixtures of frequently used UV-filters on pS2-gene transcription in MCF-7 cells

2

Marjoke Heneweer, Martine Muusse, Martin van den Berg and J. Thomas Sanderson

Institute for Risk Assessment Sciences (IRAS), Utrecht University, PO box 80176, 3508 TD Utrecht, The Netherlands

Submitted

Abstract

In order to protect consumers from ultraviolet (UV) radiation and enhance light stability of the product, three to eight UV-filters are usually added to consumer sunscreen products. High lipophilicity of the UV-filters has been shown to cause bioaccumulation in fish and humans, leading to environmental levels of UV-filters that are similar to those of PCBs and DDT. In this paper, estrogen-regulated pS2 gene transcription in the human mammary tumor cell line MCF-7 was used as a measure of estrogenicity of 4 individual UV-filters. Since humans are exposed to more than one UV-filter at a time, an equipotent binary mixture of 2-hydroxy-4-methoxy-benzophenone (BP-3) and its metabolite 2,4-dihydroxy benzophenone (BP-1) as well as an equipotent multi-component mixture of BP-1, BP-3, octyl methoxy cinnamate (OMC) and 3-(4-methylbenzylidene) camphor (4-MBC) were also evaluated for their ability to induce pS2 gene transcription in order to examine additivity. An estrogen receptor-mediated mechanism of action was expected for all UV-filters. Therefore, our null-hypothesis was that combined estrogenic responses, measured as increased pS2 gene transcription in MCF-7 cells after exposure to mixtures of UV-filters, are additive, according to a concentration-addition model.

Not all UV-filters produced a full concentration-response curve within the concentration range tested (100 nM–1 µM). Therefore, instead of using EC_{50} values for comparison, the concentration at which each compound caused a 50% increase of basal pS2 gene transcription was defined as the C50 value for that compound and used to calculate relative potencies. For comparison, the EC_{50} value of a compound is the concentration at which the compound elicits an effect that is 50% of its maximal effect. Individual UV-filters increased pS2 gene transcription concentration-dependently with C50 values of 0.12 µM, 0.5 µM, 1.9 µM and 1.0 µM for BP-1, BP-3, OMC and 4-MBC, respectively. Estradiol (E2) had a C50 value of 4.8 pM. Experiments with equipotent mixtures all supported our null hypothesis that mixtures of UV-filters act additively to activate the estrogen receptor (ER). In view of our results and observed plasma levels it cannot be excluded that daily exposure to sunscreen formulations may have estrogenic effects in humans.

Introduction

Ultraviolet (UV)-filters are organic chemicals that absorb UVA (315-400 nm) or UVB (280-315 nm) radiation. These chemicals are added to consumer sunscreen products in concentrations up to 10%.[1] They are highly lipophilic and can therefore accumulate in humans and the environment. Six different UV-filters were identified in fish in the Maarfelder Lake (Eifel, Germany) at total concentrations of 2 mg/kg lipid in perch and 0.5 mg/kg lipid in roach.[2] Both fish species were contaminated with UV-filters, PCBs and DDT at similar levels. Therefore, UV-filters should be considered relevant environmental contaminants.

Human exposure to UV-filters can occur via dermal absorption [3-5] and through the food chain, for example by consumption of contaminated fish. 2-Hydroxy-4-methoxybenzophenone (BP-3) is well absorbed via the dermal and oral routes.[6] Both BP-3 and its metabolite 2,4-dihydroxybenzophenone (benzophenone-1, BP-1) have been detected in human urine 4 h after application of commercial products to the skin.[4,7] Analyses of human milk indicate bioaccumulation of UV-filters in humans after prolonged exposure. BP-3 and/or octyl methoxy cinnamate (OMC) were present in detectable amounts.[8]

Over the past few years, the list of identified endocrine disrupting environmental contaminants has grown rapidly. Many man-made chemicals are able to bind to the estrogen receptor (ER), and subsequently elicit estrogenic effects. BP-3 was shown to be slightly estrogenic in a yeast bioassay, in which yeast was transfected with estrogen receptor α (ERα), estrogen responsive elements (ERE) and a *lac*Z reporter gene [9] and Schlumpf [10] observed various estrogenic effects after *in vitro* and *in vivo* exposure to several UV-filters. Five out of six tested UV-filters increased MCF-7 cell proliferation with EC_{50} values ranging from 1.56 to 3.73 µM, making them six orders of magnitude less potent than E2. For comparison, E2 had a EC_{50} value of 1.22 pM. pS2 is a secretorial protein from MCF-7 cells which is estrogen-regulated [11,12] and its synthesis in and secretion from MCF-7 cells was also induced by UV-filter exposure.[10] Additionally, BP-3 showed anti-androgenic activity *in vitro.*[13] *In vivo*, female immature rats that received UV-filters for 4 days in feed

showed a dose-dependent increase in uterine weight in response to 3-(4-methylbenzylidene) camphor (4-MBC), OMC and BP-3. Dermal application of 4-MBC to immature hairless rats also increased uterine weight.[10]

In this paper, pS2 gene expression in the human mammary tumor cell line MCF-7 was used as a measure of estrogenicity of 4 UV-filters. Since humans are exposed to more than one UV-filter at a time, a binary mixture (BP-1 and BP-3) and a multi-component mixture of 4 UV-filters (BP-1, BP-3, 4-MBC and OMC) were also evaluated for their ability to induce pS2-gene expression. The estrogenic effect of a mixture can be calculated on the basis of the estrogenic effects of its individual components. Non-additive interactions among multiple components of a mixture would cause a deviation from the expected additivity of the effects.[14] Two main analytical methods to predict the expected effects of a mixture, concentration-addition and response-addition, have been described. Concentration-addition assumes a similar mechanism of action of all components in the mixture.[14-16] In case of similarly acting components, there is a consensus that concentration-addition is a suitable and valid concept for the prediction of mixture effects. In contrast, response-addition assumes that the compounds act via independent pathways.[14,16] This method is unsuitable for the assessment of interactive effects among estrogenic compounds that act as ER agonists.[14,15,17] In case of sigmoidal concentration-response curves, it is incorrect to calculate expected additive effects by arithmetic summation of individual responses.[18,19] However, an ER-mediated mechanism of action was expected for all UV-filters in our test mixtures and therefore our null-hypothesis was that the model of concentration-additivity would be a suitable model to determine that the estrogenic effects of multi-component mixtures do not depart from additivity. In addition, we wanted to validate pS2 gene expression as a method to assess estrogenicity, in addition to MCF-7 cell proliferation. pS2 gene expression experiments have several advantages above cell proliferation assays because they are more rapid requiring shorter incubation times, and may in specific instances avoid non-ER mediated mitogenic effects on the cells or interferences with the MTT assay.

Materials and Methods

Chemicals

Estradiol (Sigma, E2758) was dissolved in 70% EtOH. UV-filters were obtained from Merck (Darmstadt, Germany). 4-MBC (Eusolex 6300, CAS-No 36861-47-9), OMC (Eusolex 2292, CAS-No 5466-77-3), BP-3 (Eusolex 4360, CAS-No 131-57-7) and BP-1 (CAS-No 131-56-6) were dissolved in 70% EtOH and stored at -20°C. Each compound was reported to be >98% pure by the manufacturer.

Cell culture and experimental design

The MCF-7 malignant human mammary epithelial cancer cell line was obtained from the American Type Culture Collection (ATCC No. HTB-22) and cultured in phenol red-free RPMI 1640 medium containing glutamine (GibcoBRL 11835-030), 10% heat inactivated FCS (GibcoBRL 10099-141), 1% penicillin/streptomycin (GibcoBRL 15140-114) and 1 µg/ml insulin (Sigma, St. Louis, MO, USA) in a 5% CO_2 atmosphere at 37°C. MCF-7 cells were placed on steroid-free medium (containing dextran-coated charcoal-treated FCS) 72h prior to the start of the estrogenicity assays.

Then, for MCF-7 cell proliferation experiments, cells were plated in 24-well plates containing $1x10^5$ cells and 1 ml medium per well. The following day, after cells had attached to the bottom of the wells, medium was refreshed and 1 µl of compound was added to the cells for an incubation period of 6 days. Final solvent concentration was 0.1%. After 3 days, medium and compounds were refreshed. After 6 days, an MTT-test was performed as an indicator for cell numbers, as described previously.[20]

For pS2 gene transcription experiments, cells were transferred to 12-well plates containing 2 ml steroid-free medium. The following day, after cells had attached to the bottom of the wells, medium was refreshed and 2 µl of test compounds were added to the cells for an incubation period of 24h. Final solvent concentration was 0.1%. Concentration-response curves for the induction of the pS2 gene transcription by E2 were determined at different exposure times, indicating that

an exposure of 24h was sufficient for accurate measurements (data not shown). A concentration-response curve for E2 was included in each experiment as internal control.

RNA isolation and PCR conditions

After 24h, RNA was isolated from MCF-7 cells using the RNA Instapure System (Eurogentec, Liège, Belgium) according to enclosed instructions. Purity of RNA was assessed by measuring 260/280 nm absorbance ratio. Absorbance at 260 nm was used to calculate the concentration of RNA. RNA was stored at –70°C at a concentration of 10 ng/µl. For pS2 transcripts, primers designed by Lee [21] were used. Primer sequences were 5′-GCGAAGCTTGGCCACCATGGAGAACAAGG-3′ and 5′-GCGGATCCACGAACGGTGTCGTCGAA-3′. The PCR-product for pS2 was 189 bp. Reverse transcriptase polymerase chain reactions (RT-PCR) were performed using the Access RT-PCR System (Promega, Madison, WI, USA) according to the supplied protocol. A 20 ng amount of RNA was added to the RT-PCR reaction. Annealing temperature was 55°C, extension lasted 45s at 72°C and 25 cycles were performed. Mg^{2+} concentration was 1 mM. The expected amplification product of 189 bp was detected using 2% agarose gel electrophoresis and ethidium bromide staining. Intensity of the stains was quantified using a FluorImager (Molecular Dynamics, USA). Because β-actin mRNA expression was also stimulated by estrogens, we chose 36B4 as internal control.

Effects of individual UV-filters and mixtures on pS2 gene transcription and calculation of relative potencies

First, concentration-response curves for individual compounds were established. In order to get an equipotent multi-component mixture of the 4 UV-filters, we used nonlinear regression analysis to determine the concentration at which each compound caused a 50% increase of basal pS2 gene transcription and defined this concentration as C50. Subsequently, relative potencies (RPs) were derived for each compound by dividing the C50-value for E2 by the C50-values of each single

compound. These RPs were used to calculate respective fractions of the compounds in the equipotent mixtures.

MCF-7 cells were exposed to 7 dilutions of an equipotent mixture consisting of 4 UV-filters (solvent control, 0.01x, 0.03x, 0.1x, 0.3x, 3x and 10x), to obtain a concentration-response curve for pS2 induction. For each dilution of the mixture, estrogen equivalents (EEQ, RP of individual compound x concentration in mix) was calculated. The effect on pS2 transcription was plotted against the sum of the individual EEQ values for the mixture and compared with the E2-curve. Overlapping curves suggest an additive mechanism of estrogenicity. A statistically significant shift of the EC_{50}-values obtained from the mixture-curve to the left or right, indicates an synergistic or antagonistic effect, respectively.

Additionally, we used the toxic unit approach to confirm results obtained by the EEQ-approach. Additivity can be expressed algebraically by the following equation.[19]

$$\sum_{i=1}^{n} \frac{ci}{ECxi} = 1,$$

where *n* is the number of components in a mixture, ECx_i is the concentration of the i^{th} mixture component that elicits *x%* effect when applied individually. *Ci* is the concentration of the respective component in a mixture eliciting an effect of *x%*. Each fraction (c_i/ECx_i) represents the concentration of a mixture component scaled to its relative response and is generally referred to as the toxic unit of that component.[22] When this equation results in values <1, synergism is suggested. When values >1 are observed, the components can be considered antagonists. This method can be used to analyse combinations of compounds, irrespective of the shape of their individual concentration-response curves. Also in the case of partial agonism, this method can be used.[18]

Equipotent binary mixtures of BP-1 and BP-3, based on RPs for single compounds, were added to the cells (final solvent concentration was 0.1%). The concentration of each compound when tested alone (0:100 or 100:0), was

approximately equal to the C50-value. The concentration-ratio of each binary mixture was varied, so that each mixture would produce the same effect as the C50 response of each individual component, assuming additivity. Ratios of observed and expected responses were calculated and deviations from 1 indicate synergism (ratio < 1) or antagonism (ratio > 1).

Data analysis and statistics

In each experiment, each concentration was tested in triplicate. Each independent experiment was performed 2-4 times and found to be reproducible. Each graph shows one representative experiment. Concentration-response curves were plotted using Prism 3.0 (GraphPad Software Inc. San Diego, CA, USA). All error bars represent standard errors of the mean (SEM). Statistically significant differences among means were identified using a one-way ANOVA followed by Tukey's posteriori test.

Results

pS2 gene transcription and MCF-7 cell proliferation after exposure to E2

After a six day E2 exposure, a maximum increase in cell proliferation of three-fold above basal levels was observed, which was similar to the maximum increase of 3-4 fold in pS2 gene transcription experiments where exposures were for 24 h (figure 1). Average EC_{50} values for E2 were 14 and 12 pM in cell proliferation and in pS2 gene transcription experiments, respectively. The observed difference was not statistically significant. Results with UV-filters did not indicate differences in sensitivity between cell proliferation experiments and pS2 gene transcription (data not shown).

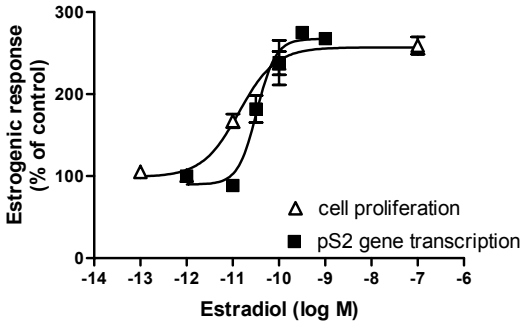

Figure 1: Estrogenic response (% of control) in MCF-7 human mammary carcinoma cells after exposure to estradiol. Δ represent data of cell proliferation experiments. ■ represent data of pS2 gene transcription experiments. Datapoints are averages of three measurements and error bars represent standard error of the mean (SEM).

Effects of individual UV-filters on E2-mediated pS2 gene transcription

Concentration-response curves for the UV-filters were obtained after a 24h exposure of MCF-7 cells to each compound, individually. Exposure of MCF-7 cells to BP-1, BP-3, 4-MBC or OMC each resulted in concentration-dependent increases of pS2-gene transcription (figure 2). Maximum increase of pS2 gene transcription was approximately 3-4 fold above control levels, which was similar to that of E2. For 4-MBC, a full concentration-response curve was not obtained.

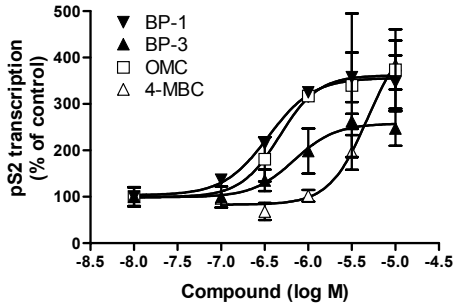

Figure 2: Concentration-response curves for induction of pS2 gene transcription in MCF-7 human mammary carcinoma cells after a 24h exposure to individual UV-filters benzophenone-1 (BP-1), benzophenone-3 (BP-3), octyl methoxy cinnamate (OMC) and 3-(4-methylbenzylidene) camphor (4-MBC). Datapoints represent averages of three measurements. Error bars represent standard error of the mean (SEM).

Therefore, to compare potencies, we determined concentrations at which each compound caused a 50% increase of basal pS2 gene transcription (C50-value). Similar approaches have been used by Soto [23] and Birkhøj [24] who also used an arbitrary effect level for comparison of potencies. For E2, BP-1, BP-3, 4-MBC and OMC, these C50 values were 4.8 pM, 0.12 µM, 0.5 µM, 1.9 µM and 1.0 µM, respectively. From these values, relative potencies (RPs) for individual UV-filters were calculated (table 1).

Table 1: Concentrations (C50) of the compounds causing a 50% increase of basal pS2 transcription levels, their potencies (RPs) relative to estradiol and concentrations of the compounds in the 1x mix.

	C50-values (M)	RP	Concentration in 1x mix (M)
E2	4.80E-12	1	
BP-3	5.00E-07	9.60E-06	1.4E-06
BP-1	1.22E-07	3.93E-05	3.5E-07
4-MBC	1.85E-06	2.59E-06	5.3E-06
OMC	1.04E-06	4.62E-06	3.0E-06

Effects of equipotent mixtures of UV-filters on E2-mediated pS2 gene transcription

When cells were dosed to dilutions of the mixture containing equipotent concentrations of BP-1, BP-3, OMC and 4-MBC, a concentration-dependent response was obtained. The maximum increase in pS2 gene transcription was about

3-fold and similar to the maximum increase obtained by E2-exposure in this experiment (figure 3). Comparison of the EC_{50} values from both curves, showed that these were not statistical significantly different from each other (P=0.1592). The sum of the toxic units of the various mixture components was calculated to be 1.35 at the C50 effect level.

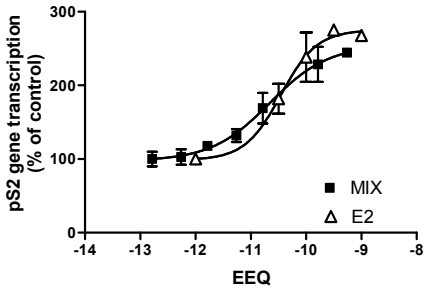

Figure 3: Concentration-dependent curves for induction of pS2 gene transcription after a 24h exposure of MCF-7 human mammary carcinoma cells to estradiol (E2) or different dilutions of an equipotent mixture of UV-filters (MIX). Dilutions of the mixture were expressed in estradiol equivalents (EEQs) for comparison with E2. Datapoints represent averages of three measurements. Error bars represent standard error of the mean (SEM).

An equipotent binary mixture of BP-1 and BP-3, based on their respective RP, was tested in the MCF-7 cell line for effects on induction of pS2-gene expression (table 2). The concentration-ratio was varied and the concentration of each compound alone (0:100 or 100:0), was approximately equal to the C50-value. No statistically significant differences between observed and expected responses were observed (figure 4).

Table 2*:* Concentrations of BP-1 and BP-3 in equipotent binary mixtures with varying concentration-ratio. The concentration of each component alone was equal to the concentration (C50) that produces a 50% increase of pS2 transcription above control levels.

(BP-1:BP-3)	BP-1 (M)	BP-3 (M)
0:100	0	5.0E-07
20:80	2.4E-08	4.0E-07
40:60	4.9E-08	3.0E-07
60:40	7.3E-08	2.0E-07
80:20	9.8E-08	1.0E-07
100:0	1.2E-07	0

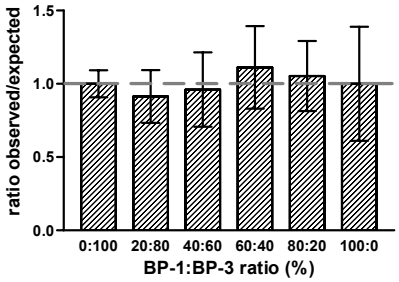

Figure 4: Ratio between observed and expected effects on pS2 transcription after exposure of MCF-7 human mammary carcinoma cells to equipotent mixtures of benzophenone-1 (BP-1) and benzophenone-3 (BP-3) in various ratios. The concentration of each component alone was equal to the concentration (C50) that produces a 50% increase of pS2 transcription above control levels. Bars represent averages of three measurements. Error bars represent standard error of the mean (SEM).

Discussion

Comparison of pS2 gene transcription and MCF-7 cell proliferation as endpoint for estrogenicity

EC_{50} values for E2 were 14 pM in an *in vitro* MCF-7 cell proliferation experiment. The average EC_{50} value, obtained from several pS2 gene transcription experiments, was 12 pM. Both endpoints had similar maximum estrogenic responses after E2-exposure, since a 3-fold increase above basal levels in cell number in the cell proliferation experiment and in pS2 gene transcription were observed. Therefore, we conclude that pS2 gene transcription is a suitable alternative to cell proliferation for assessing estrogenicity in MCF-7 cells.

Comparison of estrogenic potencies of UV-filters

The pS2 gene was originally identified as an estrogen-inducible transcript in MCF-7 cells [11] and pS2-gene expression is a suitable marker for assessing the estrogenicity of various compounds.[23] To study the estrogenicity of mixtures of UV-filters, pS2 gene transcription inducing potencies of single compounds and mixtures were determined from concentration-response experiments. They were compared to the potency of estradiol (E2) in order to express the biological potency of the UV-filters in E2-equivalents under these specific conditions. For one of the compounds (4-MBC), concentration-response curves could not be determined completely. Consequently, EC_{50} values could not be used as a good measure of relative potency. Therefore, in order to perform experiments with equipotent mixtures we had to use other than the EC_{50} value, for which we chose the C50 value (50% induction of pS2 gene transcription above background levels).

Based on RPs obtained from our study, the order of estrogenic potency was E2 >> BP-1 > BP-3 > 4-MBC > OMC, with E2 being approximately 5 orders of magnitude more potent than the UV-filters. Jorgensen and coworkers have also described effects on pS2 gene transcription.[25] They showed that bisphenol A, methoxychlor, endosulfan and dibutylphthalate, which are widely recognized as environmental estrogens, are also 5 to 6 orders of magnitude less estrogenic than

E2. Therefore, the UV-filters that were tested in this study fall within the same range of estrogenic potencies and may also be considered environmental estrogens.

Both BP-3 and 4-MBC were shown to increase pS2 secretion in MCF-7 cells.[10] Our results showing increased pS2 gene transcription, confirm this observation. However, OMC did not increase pS2 secretion in MCF-7 cells at a concentration of 10 μM, [10] in contrast to our observed 3-4 fold increase in pS2 gene transcription at the same concentration. Possibly, estrogenic properties of OMC are strong enough to elicit pS2 gene transcription after 24h, but too weak to stimulate pS2 protein synthesis and secretion, even after an incubation time of 72h.

EC_{50} values obtained from a cell proliferation experiment identified OMC to be slightly more potent than 4-MBC.[10] In the same study, BP-3 was the only UV-filter to elicit a maximal response that was similar to that of E2. Estrogenicity of UV-filters has not only been found in *in vitro* studies, but also in *in vivo* experiments. A rat uterine growth experiment after oral UV-filter administration showed 4-MBC to be more potent than OMC, which was in turn more potent than BP-3.[10]

Equipotent binary mixture of BP-1 and BP-3

Assuming additivity, all different concentration-ratios in our *in vitro* study with an equipotent binary mixture of BP-3 and BP-1 would produce the same effect. As no statistically significant differences between observed and expected responses were observed, we suggest that both compounds act additively to stimulate estrogenicity, confirming our null-hypothesis.

In vitro, BP-3 acted as a full ER agonist, being 5 orders of magnitude less potent than E2. However, *in vivo*, maximum BP-3-mediated increase of uterine weight in immature rats was not as large as after exposure to ethynyl estradiol.[10] This observation suggests metabolic deactivation of BP-3 within the animal. BP-3 passes through the skin in significant amounts varying from 10% [5] to 35% [26] of applied dose. However, in contrast to BP-3 itself, one of its main metabolites, BP-1, binds to the ER in the micromolar range.[27] BP-1 and another benzophenone metabolite, 4-hydroxybenzophenone, both elicited estrogenic effects in a yeast two-hybrid assay in which yeast cells were transfected with two expression plasmids containing an

estrogen receptor ligand binding domain and a β-galactosidase reporter gene, with a potency 100 fold greater than that of BP-3.[28] Results from our study also indicate that BP-1 (C50 = 0.1 μM) is more estrogenic than BP-3 (C50 = 0.5 μM). These results clearly indicate that metabolism of BP-3 can lead to significant bioactivation besides deactivation to possible less estrogenic compounds. Deactivation has previously been confirmed for rats and humans.[29]

Equipotent multi-component mixture of BP-1, BP-3, OMC and 4-MBC

Exposure of MCF-7 cells to different dilutions of an equipotent mixture of 4 UV-filters resulted in a concentration-dependent increase in pS2 gene transcription with a maximal increase similar to that elicited by E2. After mathematical transformation of the dilutions of the mixture to estrogen equivalents (EEQs), both curves overlapped with similar maximum effects and EC_{50} values that did not differ statistically significantly, not rejecting our null hypothesis that mixtures of UV-filters act additively on the ER. The C50 value of the multi-component mix corresponded with a 0.12 dilution of the multi-component mix.

In order to predict responses to a multi-component mixture of 4 UV-filters using the model of concentration-addition, it is assumed that all chemicals have similar mechanism of action, resulting in parallel concentration-response curves with similar shape. Our experimental results showed a different slope and efficacy for BP-3. However, this phenomenon is not unique and observed both for compounds that are easily biotransformed such as PAHs and biopersistent compounds such as dioxins and PCBs. As MCF-7 cells contain significant CYP1A1 and 1B1 activity, it is likely that this enzyme activity is also involved in hydroxylation of UV-filters, significantly influencing the final ER-mediated response.

Metabolism may be a confounding factor in determining concentration additivity of mixtures that are composed of different compounds. However, consistent with the assumption that these estrogenic UV-filters act through the same mode of action, concentration additivity was observed in our present study (Fig 3; Fig 4), in spite of the experimental limitations described above. Furthermore, the toxic unit approach also supported that our mixture components interacted in a close to

additive manner. The overall conclusion is that our experiments indicate that the tested UV filters, under these experimental conditions, do follow the concentration additivity concept for compounds with a similar mechanism of action.

Biological relevance

The C50-value of the multi-component mixture equals concentrations of 0.21, 0.05, 0.78 and 0.44 µM for BP-3, BP-1, 4-MBC and OMC, respectively. The total concentration of estrogenic UV-filters in fish ranged from 1.6 to 7.8 µM in fat, or from 0.02 to 0.2 µM in whole fish.[10] The sum of the 4 concentrations in our undiluted mixture falls well within the range of concentrations presently found in fish. Furthermore, BP-3, 4-MBC and OMC can be found in a high number of commercial UV-screens in Europe.

75% or less of the skin surface is, often daily, treated with sunscreens during a period of 3 to 4 weeks in summer.[30] For a number of these UV-filters this application is sufficient to cause bioaccumulation in the human body. Maximum plasma levels of BP-3, 4-MBC and OMC in postmenopausal women were 200 ng/ml (0.9 µM), 20 ng/ml (0.1 µM) and 10ng/ml (0.03 µM), respectively, 3-4 hours after application of a formulation containing 10% (wt/wt) of each compound.[31] The C50-values of 0.5, 1.9 and 1 µM for BP-3, 4-MBC and OMC, respectively, fall within the range of the reported plasma levels after daily exposure to sunscreens. In view of our results and observed plasma levels it cannot be excluded that daily exposure to sunscreen formulations may have estrogenic effects in humans. The observed estrogenic effects of these commercially available cosmetic compounds in human breast carcinoma cells requires further investigations, particularly in relation to the development of estrogen-responsive breast tumors in high risk women.

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A comparison of human H295R and rat R2C cell lines as *in vitro* screening tools for effects on aromatase

3

Marjoke Heneweer, Martin van den Berg, J. Thomas Sanderson

Institute for Risk Assessment Sciences (IRAS), Utrecht University, PO Box 80176, 3508 TD Utrecht, The Netherlands

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Abstract

In this study we evaluated the rat Leydig cell carcinoma cell line R2C and the human adrenocorticocarcinoma cell line H295R for their suitability as *in vitro* screening tools for potential interference of xenobiotics with aromatase activity. A 24h exposure to prochloraz (PRO), fadrozole (FAD) and epoxyconazole (EPO) resulted in complete catalytic inhibition in H295R and R2C cells. In H295R cells, PRO and FAD were mixed-type inhibitors with apparent K_i values of 0.04 μM and 0.03 μM, and apparent K_i' values of 0.33 μM and 0.06 μM, respectively. EPO was a competitive inhibitor with an apparent K_i value of 0.51 μM. In R2C, all three compounds showed mixed type inhibition kinetics, with apparent K_i values (μM) of 0.004, 0.003 and 0.07, and apparent K_i' values (μM) of 0.41, 0.01 and 2.42, respectively.

Exposure for 24h of H295R cells to 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP), prostaglandin E2 (PGE2), phorbol 12-myristate 13-acetate (PMA), or dexamethasone (DEX) resulted in 4.0, 2.8, 3.6 or 3.6-fold induction of aromatase activity, respectively, as well as in an increase of several human aromatase transcripts with promoter-specific 5'-ends (pII and I.3). In R2C cells, only PMA slightly induced aromatase activity. A 24h exposure of H295R cells to atrazine (ATR), resulted in a 3-fold induction of aromatase activity and a slight increase in pII and I.3 aromatase transcripts. However, ATR did not induce aromatase activity in R2C cells. We conclude that the H295R cell line contains aromatase promoter regions, which are responsive to the respective stimulants. They play a role in aromatase regulation in various tissues such as brain, placenta, healthy and diseased gonadal and breast tissue and therefore they may play an important role in tumor genesis, development, behavior and reproduction. The H295R cell line may therefore be a relevant and useful tool in risk assessment of xenobiotics. The R2C cell line, although not suitable for studying induction, appears to be a more sensitive cell line for studying inhibitory effects of xenobiotics on aromatase activity.

Introduction

Estrogens are largely responsible for the development of sexually dimorphic anatomical, functional and behavioral characteristics that are essential for reproduction in vertebrates. Moreover, they have been shown to play an important role in the etiology or progression of several diseases, including breast cancer.[1] In the past, research on endocrine disruptors has mainly focused on the interaction of chemicals with hormone receptors. More recently, interactions of environmental contaminants with enzymes that play a role in estrogen synthesis have also become a focus of research.

A key enzyme in the synthesis of estrogens is the cytochrome P450 enzyme aromatase (CYP19). CYP19 is responsible for the conversion of androstenedione into estrone (E1), or testosterone (T) into 17β-estradiol (E2), the main estrogens in humans. In humans, adrenal tumors can cause a moderate overexpression of aromatase, which has been associated with severe feminization in boys.[2] Elevated aromatase activity has also been associated with increased risk of breast cancer development.[3-6]

Aromatase is present in many different cell types and species throughout the entire vertebrate phylum. In invertebrates aromatase activity is found in gonad tissue and brain, but in humans this enzyme activity can also be detected in the placenta, adipose tissue, and fetal liver.[2,3,7,8] There is only one gene for aromatase, but its expression is regulated in a species- and tissue-specific manner by differential use of several promoters.[5,9] In healthy human mammary tissue, aromatase expression is regulated by promoter region I.4, which is under control by glucocorticoids. In human tissues such as ovary, it is regulated by promoter regions pII and I.3 via the phosphorylation pathway PKA.[1]

Previous studies suggest that several compounds can interact with the CYP19 enzyme as inhibitors [10-12] or inducers.[10,12,13] Based on molecular structure and previous studies with atrazine (ATR) and prochloraz (PRO),[12] carbendazim (CAR), epoxyconazole (EPO) and imidacloprid (IMI) were suspected to interfere with aromatase activity and selected for the present study.

ATR is a triazine herbicide that is widely used in agriculture. Exposure has been associated with an increase in plasma estrogen levels in rats.[14,15] Additionally, an increased risk of ovarian cancer in female farm workers in Italy [16] and an increased risk of breast cancer in the population of Kentucky, USA [17] have been observed that might have been related to ATR exposure. Recent studies have shown that ATR can induce aromatase activity in certain human cell systems *in vitro.*[10] Studies have shown that exposure to CAR leads to testicular toxicity and abnormal spermatogenesis in rats.[18-20] EPO and PRO are both azole fungicides that have been listed as potential endocrine disrupters by affecting the sex hormone balance and pituitary weight, respectively in rats.[21] PRO is widely used in western countries within horticulture and agriculture. *In vitro*, PRO acted as an estrogen and androgen receptor antagonist.[22] Furthermore, EPO has been associated with an increase in ovarian tumors [21] and *in vivo* oral exposure of rats to PRO resulted in anti-androgenic effects such as reduced weights of the ventral prostate and seminal vesicles.[23]

The main goal of this study was to compare two cell lines for their suitability as *in vitro* screening tools for potential interference of xenobiotics with aromatase expression and catalytic activity. With aromatase regulation being highly species- and tissue specific and rats being a commonly used model in endocrine disruption studies while humans being a relevant target species, we choose to use an aromatase expression cell line from each species. We compared a rat Leydig cell carcinoma (R2C) and a human adrenocorticocarcinoma (H295R) for their suitability as *in vitro* screening tools for effects on aromatase expression and catalytic activity.

Methods

Cell culture

H295R [24] cells were obtained from the American Type Culture Collection (ATCC # CRL-2128) and grown in 1:1 Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12) containing 365 mg/ml L-glutamine and 15 mM HEPES (GibcoBRL 31300-038). The culture medium was supplemented with 10 mg/l insulin, 6.7 μg/l sodium selenite and 5.5 mg/l transferrin (ITS-G, GibcoBRL 41400-045), 1.25 mg/ml bovine serum albumin (Sigma A9647), 100 U/l penicilline/100 μg/l streptomycin (GibcoBRL 15140-114) and 2% steroid-free replacement serum Ultroser SF (Sopachem, France). R2C cells were a generous gift from Prof. dr. M.A. Blankenstein, University Medical Center Utrecht, The Netherlands and grown in Ham's F-10 nutrient mix with Glutamax-I, containing 217 mg/l L-Alanyl-L-Glutamine (GibcoBRL 41550-021). The medium was supplemented with 15% horse serum, 2.5% fetal calf serum and 100 U/l penicilline/100 μg/l streptomycin. Serum was not heat inactivated.

Cell treatment

Cells were cultured in 24-well tissue culture plates (Greiner, Alphen a/d Rijn, The Netherlands) at 37°C and 5% CO_2.

All pesticides were obtained from Riedel-deHaën (Zwijndrecht, The Netherlands). ATR, EPO, IMI and PRO were dissolved in DMSO (Sigma-Aldrich, Zwijndrecht, The Netherlands). CAR was dissolved in methanol (MeOH, Rathburn Chemicals Limited, Walkerburn, Scotland). Negative control cells were exposed to 1 μl DMSO or MeOH (final concentration of 0.1% in medium).

In order to study different routes of induction, cells were exposed to various concentrations of several stimulants of second messenger pathways, such as 8-Br-cAMP, phorbol 12-myristate 13-acetate (PMA), prostaglandin E2 (PGE2) and dexamethasone (DEX) for 24 hours. All compounds were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and dissolved in DMSO except for DEX and PGE2, which were dissolved in EtOH.

Inductive or inhibitory effects of chemicals were studied by adding 1 μl of various chemicals to 24-well plates containing 1 ml of cell culture medium per well. Final concentrations ranged from 0 to 100 μM . Fadrozole (FAD), kindly provided by Novartis Pharma AG (Basel, Switzerland), was dissolved in DMSO and used as a positive control for aromatase inhibition.[25,26]

Measurement of aromatase activity and total protein

The catalytic activity of aromatase was determined by the tritiated water release assay as previously described.[13,27] The specificity of this radiometric assay in H295R cells was verified by measuring the production of estrone and by using 4-hydroxyandrostenedione, a specific irreversible inhibitor of the catalytic activity of aromatase.[13] 4-Hydroxyandrostenedione was able to block tritiated water release completely at 0.5 μM in R2C cells indicating the assay to be specific for aromatase activity in this rat Leydig cell line. Cells were exposed to pesticides for 24 hours prior to the aromatase assay. H295R and R2C cells were exposed to 54 nM and 25 nM 1β-^{3}H-androstenedione (New England Nuclear Research Products, USA), respectively, which was added to 24-well plates containing 250 μl serum free culture medium and incubated for 1.5 h at 37°C, 5% CO_2. Further steps were carried out as described previously.[10,28] An assay based on the method of Lowry [29,30] was used to correct for differences in protein content of the wells.

Promoter-specific mRNA levels

RNA was isolated from H295R cells after 24h exposure using the RNA Instapure System (Eurogentec, Liège, Belgium) according to the enclosed instruction. Purity of RNA was checked by measuring 260/280 nm absorbance ratio. RNA was stored in –70°C in aliquots of 50 ng/μl.

Primers were designed for specific aromatase promoter regions I.3, pII and I.4 (table I). RT-PCR was performed using the Access RT-PCR System (Promega, Madison, WI, USA) according to supplied protocol, except for the use of an annealing temperature of 61°C for I.3 and pII, 1 minute extension and 35 cycles. Amplification products were detected using 2% agarose gel electrophoresis and ethidium bromide

staining. Intensity of the stains were quantified using a FluorImager (Molecular Dynamics, USA). As an internal control, RT-PCR reactions amplifying the housekeeping gene β-actin were included for each RNA sample. For β-actin amplification, Mg^{2+} concentration was 2 mM, annealing temperature was 54°C, and 25 cycles were performed.

Specificity of the I.3 and pII primers was assessed by sequencing of the amplification product (BaseClear, Leiden, The Netherlands) after extracting the DNA from the agarose gel using QIAEX II Agarose Gel Extraction kit (Qiagen GmbH, Hilden, Germany) according to supplied instructions. The sequence of promoter region I.4 5'-UTR was constructed using NCBI sequence D21240 and M22246. The sequence for β-actin was obtained using NCBI sequence X00351 (Table 1).

MTT reduction assay

Mitochondrial capacity to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to blue-colored formazan was assessed as a measure of cell viability using the method of Denizot and Lang.[31] The cells in each well of the 24-well plate were exposed to 0.5 ml MTT solution containing 0.5 mg of MTT dissolved in 1 ml serum-free medium and incubated for 30 minutes at 37°C. After incubation, the MTT solution was removed and cells were washed twice with PBS. The formazan formed in the cells was extracted by lysing the cells with 1 ml of isopropanol. After an incubation period of 10 minutes at room temperature, the isopropanol was transferred to a plastic cuvette for spectrophotometric analysis (Shimadzu UV-160A, Shimadzu Benelux, Belgium) at an absorbance wavelength of 560 nm.

table 1: Primers, expected product size and 5'-ends of amplified transcripts

	Forward primer (5'-3')	Reverse primer (5'-3')	Expected product size	5'- ends of transcripts (5'-3')
I.3	GCTGCAATTCAAGCCAAAAG	GCACGATGCTGGTGATGTTATA	187 bp	TGCTGCAATTCAAGCCAAAAGATCTTTCTTGGGCTTCCTTGTTTTGACTTGTAACCATAAATTAGTCTTGCCTAAATGTCTGATCACATTATAAAACAGACTCTAAATTGCCCCCTCTGAGGTCAAGGAACACAAGATGGTTTTGGAAA
pII	TCTGTCCCTTTGATTTCCACAG	GCACGATGCTGGTGATGTTATA	112 bp	TCTGTCCCTTTGATTCCACAGGACTCTAAATGCCCCCTCTGAGGTCAAGGAACACA
I.4	GGCTCCAAGTAGAACGTGACCAACTG	CAGCCCAAGTTTGCTGCCGAA	475	GGCTCCAAGTAGAACGTGACCAACTGGAGCCTGACAGGAGGTCCCTGGCACTGGTCAGCCCATCAAACCAGGACTCTAAATTGCCCCCTCTGAGGTCAAGGAACACAAGATGGTTTTGGAAATGCTGAACCCGATACATTATAACATCACCAGCATCGTGCCTGAAGCCATGCCTGCTGCCACCATGCCAGTCCTGCTCCTCACTGGCCTTTTTCTCTTGGTGTGGAATTATGAGGGCACATCCTCAATACCAGGTCCTGGCTACTGCATGGGAATTGGACCCTCATCTCCCACGGCAGATTCCTGTGGATGGGGATCGGCAGTGCCTGCAACTACTACAACCGGGTATATGGAGAATTCATGCGAGTCTGGATCTCTGGAGAGGAAACACTCATTATCAGCAAGTCCTCAAGTATGTTCCACATAATGAAGCACAATCATTACAGCTCTCGATTCGGCAGCAAACTTGGGCTG
β-actin	AAACTACCTTCAACTCCA	ATGATCTTGATCTTCATTGT	163 bp	AAACTACCTTCAACTCCATCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACGCCAACACAGTGCTGTCTGGCGGCACCACCATGTACCCTGGCATTGCCGACAGGATGCAGAAGGAGATCACTGCCCTGGCACCCAGCACAATGAAGATCAAGATCAT

Data analysis

Enzyme inhibition can be described as competitive or non-competitive. Compounds that bind to the substrate-binding site of the enzyme are called competitive inhibitors. This type of binding results in an increased K_M value without affecting V_{max}. The apparent inhibitor dissociation constant (K_i) for the enzyme was determined by plotting apparent K_m values versus inhibitor concentration, to obtain information on the potency of the inhibitor.

Compounds binding to allosteric sites of the enzyme cause conformational changes of the molecule resulting in decreased enzyme activity. This type of binding is called non-competitive, inferring that the substrate cannot replace the inhibitor. This results in a decreased V_{max}, without affecting K_M. Some compounds act both as competitive and non-competitive inhibitors, depending on the concentration. Exposure to these compounds results in a mixed-type inhibition with increased K_M values and decreased V_{max} values. In this case, the apparent K_i and apparent inhibitor dissociation constant K_i' for the enzyme-substrate complex, were estimated from the linear parts of the slopes obtained by plotting K_m/V_{max} and $1/V_{max}$, respectively, versus inhibitor concentration to obtain information on the potency of the inhibitor.

In order to elucidate the mechanism of aromatase inhibition, typical enzyme kinetic experiments were performed using different concentrations of ^{3}H-androstenedione (25-1000 nM) and inhibitor (0.3-30 μM).

All responses are presented as means with standard error of the mean (SEM) (*n=3*). Statistically significant differences among means were determined by performing an one-way ANOVA, followed by a Dunnett's Multiple Comparison Test [32,33] at a significance level of 0.05. Non-linear regression was performed using Prism 3.0 (GraphPad Software Inc. San Diego, CA, USA) in order to calculate K_m and V_{max} values.

Results

Aromatase inhibitors

Exposure to the selective aromatase inhibitor FAD resulted in a concentration-dependent decrease of aromatase activity in both cell lines (figure 1). Results of studies on enzyme kinetics indicated a mechanism of inhibition that was mixed-type. H295R cells appeared to be almost an order of magnitude less sensitive to inhibition by FAD than R2C cells. In H295R, apparent K_i and K_i' were determined to be 0.03 μM and 0.06 μM, respectively. In R2C cells, FAD had an apparent K_i and K_i' of 0.003 and 0.01 μM, respectively.

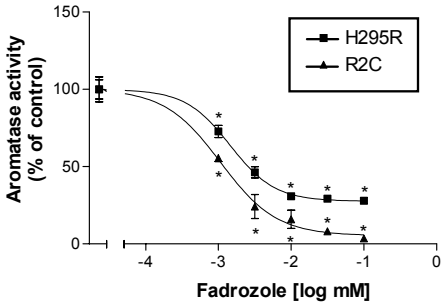

Figure 1: Aromatase activity in the human adrenocortical cell line H295R and the rat Leydig tumor cell line R2C cells after exposure to fadrozole (FAD). * Significantly different from control (P<0.05). Error bars represent SEM. Basal aromatase activities in H295R cells and R2C cells were 4±2 and 49±9 pmol/h/mg protein respectively.

Exposure of both cell lines to PRO in concentrations up to 100 μM resulted in complete catalytical inhibition of the aromatase enzyme (figures 2A and 2B).

H295R cells were again less sensitive than R2C cells for aromatase inhibition by PRO, with apparent K_i values 10 times greater than in R2C cells. Enzyme kinetic studies revealed PRO to be a mixed type inhibitor with apparent K_i = 0.04 μM and apparent K_i' = 0.33 μM in H295R cells, as described previously [12]. In R2C cells, an apparent K_i of 0.004 μM and an apparent K_i' of 0.41μM was determined (figure 3A). Exposure to EPO also resulted in complete inhibition of aromatase activity in both cell lines (figure 2A and 2B). However, enzyme kinetics studies showed that exposure of H295R cells to EPO resulted in a linear increase of K_M and no change of V_{max} indicating a competitive mechanism of enzyme inhibition with a apparent K_i of 0.51 μM (figure 3B).

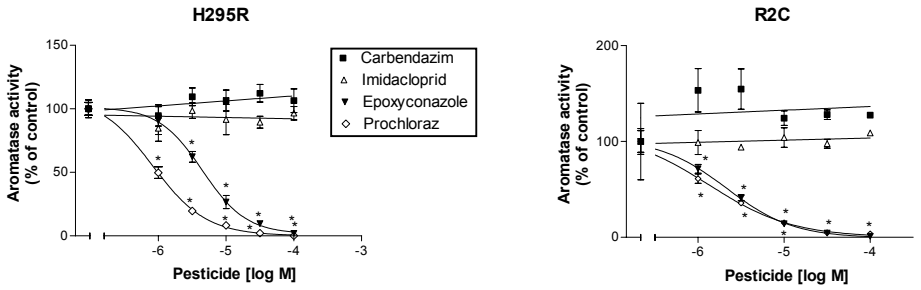

Figure 2: Left: Aromatase activity in the human adrenocortical cell line H295R cells after exposure to several pesticides. * Significantly different from control (P<0.05). Error bars represent SEM. Basal aromatase activity in H295R cells was 4±2 pmol/h/mg protein.
Right: Aromatase activity in the rat Leydig tumor cell line R2C cells after exposure to several pesticides. * Significantly different from control (P<0.05). Error bars represent SEM. Basal aromatase activity in R2C cells was 49±9 pmol/h/mg protein.

Exposure of R2C cells to EPO resulted in a mixed type inhibition of aromatase activity with an apparent K_i of 0.07 μM and an apparent K_i' of 2.42 μM. First signs of cytotoxicity were observed at an EPO concentration of 100 μM with a decrease in cell viability of 10% and 60%, in H295R and R2C, respectively. Measurements obtained at cytotoxic concentrations were excluded from further calculations. Furthermore, IC_{50} values were determined to be 2 μM for R2C cells and 4.5 μM for H295R cells, which make R2C cells the more sensitive cell line to EPO exposure.

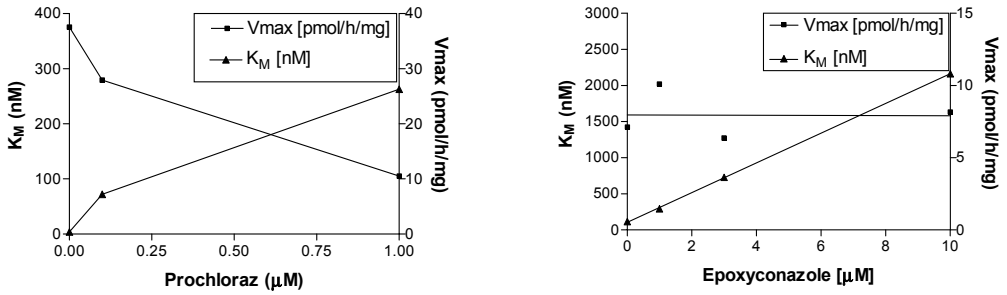

Figure 3: Left: Effect of the inhibitor prochloraz on the apparent Michaelis-Menten constant (K_M) and maximum velocity (Vmax) of aromatase in R2C cells. Exposures were for the duration of the catalytic assay only (1.5 h). Each point represents the average of two measurements. One of 3 experiments is shown.

Right: Effect of the inhibitor epoxyconazole on apparent K_M and Vmax of aromatase in H295R cells. Exposures were for the duration of the catalytic assay only (1.5 h). Each point represents the average of two measurements. One of 3 experiments is shown.

Aromatase inducers

H295R cells were exposed to 8-Br-cAMP (100 μM), DEX (50 and 100 nM), PMA (1 nM and 1 μM) and PGE2 (100 nM and 1 μM). Exposure to 8-Br-cAMP resulted in a 4-fold increase in aromatase activity (figure 4). Exposure to DEX-, PMA- and PGE2- increased aromatase activity in a concentration-dependent matter with 3.6, 3.6 and 2.8 fold induction respectively at the highest tested concentrations (figure 4). In contrast with the results obtained in the H295R cell line, the R2C cells were much less responsive to inducers of aromatase with only PMA causing a statistically significant 1.5-fold induction at the highest tested concentration (figure 4).

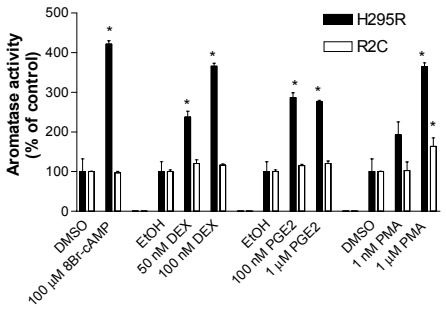

Figure 4: Aromatase activity in the human adrenocortical cell line H295R and the rat Leydig tumor cell line R2C cells after a 24h exposure to the pharmacological inducers 8-Br-cAMP, dexamethasone (DEX), prostaglandin E2 (PGE2) or phorbol 12-myristate 13-acetate (PMA). * Significantly different from control (P<0.05). Error bars represent SEM. Basal aromatase activities in H295R cells and R2C cells were 4±2 and 49±9 pmol/h/mg protein respectively.

Exposure of H295R cells to ATR at concentrations ranging from 0.3-30 µM resulted in a concentration-dependent increase in aromatase activity, resulting in 3-fold induction at 30 µM. The lowest observed effect concentration was 10 µM. However, in R2C cells ATR did not have a significant effect on aromatase activity (figure 5). ATR had no effect on cell viability in R2C cells (data not shown) after 24 hours.

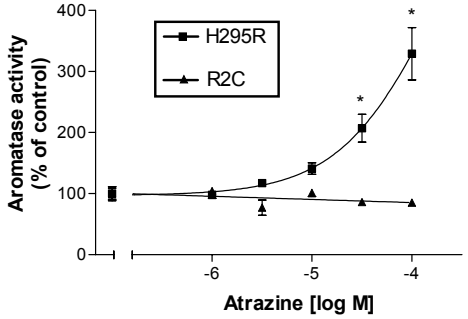

Figure 5: Aromatase activity in the human adrenocortical cell line H295R and the rat Leydig tumor cell line R2C cells after exposure to ATR. * Significantly different from control (P<0.05). Error bars represent SEM. Basal aromatase activities in H295R cells and R2C cells were 4±2 and 49±9 pmol/h/mg protein respectively.

Exposure of H295R and R2C cells to 1.0 – 100 µM of the fungicides CAR or IMI did not result in any significant change in aromatase activity after 24 hours (figure 2A,B). A slight decrease in cell viability of 20 and 40% was observed in R2C and H295R respectively at the highest tested concentration of 100 µM (data not shown).

A 24h exposure of H295R cells to 8-Br-cAMP (100 μM), PMA (1 μM), PGE2 (1 μM) and ATR (30 μM) resulted in increased levels of I.3 - and pII containing transcripts. DEX (100 nM) did not cause this increase (figure 6).

Transcripts of promoter region I.4 were not found in H295R cells. However, in primary human mammary fibroblasts, the promoter I.4 specific primers resulted in the expected amplification product (unpublished data). When increased amounts of RNA (within the range of 0.3 to 100 ng) were used in the PCR reaction, product intensity increased proportionally for all three primer pairs (data not shown).

We observed no induction of aromatase activity in R2C cells after exposure to 8-Br-cAMP. Since basal aromatase activity in R2C cells is very high, it is possible that in this specific cell line, one or more aromatase promoter regions are fully activated, leading to a constitutively high aromatase expression. The maximally induced aromatase activity leaves no room for further induction by chemicals. Our results are in agreement with other studies that concluded that aromatase activity in R2C cells was not responsive to cAMP, but instead appeared to be constitutively active at a maximal level.[39-43]

Exposing R2C cells to ATR concentrations ranging from 0-30 μM, did not result in significant effects on aromatase activity after 24 hours.

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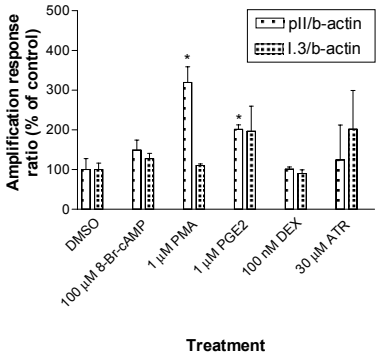
Figure 6: Levels of promoter I.3 and pII specific CYP19 mRNA in H295R cells exposed for 24 hours to DMSO vehicle, 100 μM 8-bromo-cAMP (8-Br-cAMP), 1 μM phorbol 12-myristate 13-acetate (PMA), 1 μM Prostaglandin E2 (PGE2), 100 nM dexamethasone (DEX), and 30μM atrazine (ATR). Each treatment was tested in triplicate. * Significantly different from control ($P<0.05$). Error bars represent SD.



Discussion

Aromatase inhibition in H295R and R2C cells

Competitive inhibition of enzyme activity occurs when a compound binds to the catalytic site of the enzyme. Binding of the compound to allosteric sites of the enzyme causes non-competitive inhibition. Non-competitive or mixed-type inhibition does not necessarily imply irreversibility and reversible inhibition does not imply competitiveness.[34]

Enzyme kinetics studies indicate that in H295R cells EPO inhibits aromatase activity in a competitive manner. The same was true for various other triazole compounds that were previously tested in H295R cells.[12] FAD and PRO are both imidazole-like compounds. PRO was shown to demonstrate mixed-type inhibition of aromatase activity in H295R cells.[12,35] Additionally, the imidazole-containing fungicide imazalil showed mixed-type inhibition of aromatase activity in H295R cells with apparent K_i and K_i' values of 0.015 and 0.37 μM respectively.[12] Yue and Brodie found that FAD bound tightly to aromatase at a site distinct from the steroid binding site, which supports the hypothesis that imidazole-containing chemicals show mixed-type inhibition of aromatase activity.[36]

Comparison of apparent K_i values in R2C cells indicates the following inhibitory potencies: FAD (0.003 μM) ≈ PRO (0.004 μM) > EPO (0.07 μM). EPO is an approximately 20 times less potent aromatase inhibitor than FAD and PRO. Additionally, binding of EPO to allosteric sites may also play a minor role in inhibition of aromatase activity (apparent K_i' value of 2.42 μM) in R2C cells.

Comparison of apparent K_i values of different chemicals in H295R and R2C cells show that R2C cells are approximately 10 times more sensitive towards aromatase inhibition than H295R cells, under our experimental conditions.

Aromatase induction in H295R and R2C cells

A 24h exposure of H295R cells to ATR resulted in induced aromatase activity. However, simultaneous addition of ATR and the aromatase substrate androstenedione to the H295R cells did not result in an increase in aromatase

activity. Therefore, it has been suggested that ATR is likely to exert its aromatase stimulating effect in H295R cells by increasing mRNA synthesis, which may established via elevated intracellular cAMP levels.[12,13] A 24h exposure of H295R cells to ATR resulted in increased amplification response of I.3- as well as pII-containing aromatase transcripts. Promoter regions pII and I.3 are cAMP-responsive and exposure of H295R cells to 8-Br-cAMP directly resulted in increased amplification response of I.3- as well as pII-containing transcripts. This is another indication that ATR mediated aromatase induction is established via intracellular cAMP levels. Due to the lack of aromatase induction after a 3h ATR exposure,[13] it is unlikely that interference with allosteric factors or other posttranslational events, which are generally rapid,[37] play a role in enhanced aromatase activity.

The aromatase gene consists of 10 exons (I-X). Exon II-X contain the translation starting site ATG and the coding sequence for the aromatase protein. The first exon (approximately 100 kb) is noncoding and contains several promoter regions, responding to various second messenger pathways. Certain chemicals modulate nuclear transcription factors that can enhance gene transcription by binding to stimulatory elements in the promoter region of the gene (figure 7). The tissue – and species-specific regulation of aromatase activity could be the cause of the difference in response towards known aromatase inducers between the two cell lines. The R2C Leydig cell line originates from the rat testis, where the site of aromatase expression is age-related.[1] Aromatase activity, CYP19 transcripts and protein are found predominantly in Sertoli cells before puberty and in Leydig cells in adults. In adults, aromatase expression is most probably under control of promoter pII.[38] The region upstream from exon pII contains cis-acting elements that are crucial for cAMP-mediated stimulation of promoter activity.[1]

We observed no induction of aromatase activity in R2C cells after exposure to 8-Br-cAMP. Since basal aromatase activity in R2C cells is very high, it is possible that in this specific cell line, one or more aromatase promoter regions are fully activated, leading to a constitutively high aromatase expression. The maximally induced aromatase activity leaves no room for further induction by chemicals. Our results are in agreement with other studies that concluded that aromatase activity in

R2C cells was not responsive to cAMP, but instead appeared to be constitutively active at a maximal level.[39-43] Exposing R2C cells to ATR concentrations ranging from 0-30 μM, did not result in significant effects on aromatase activity after 24 hours.

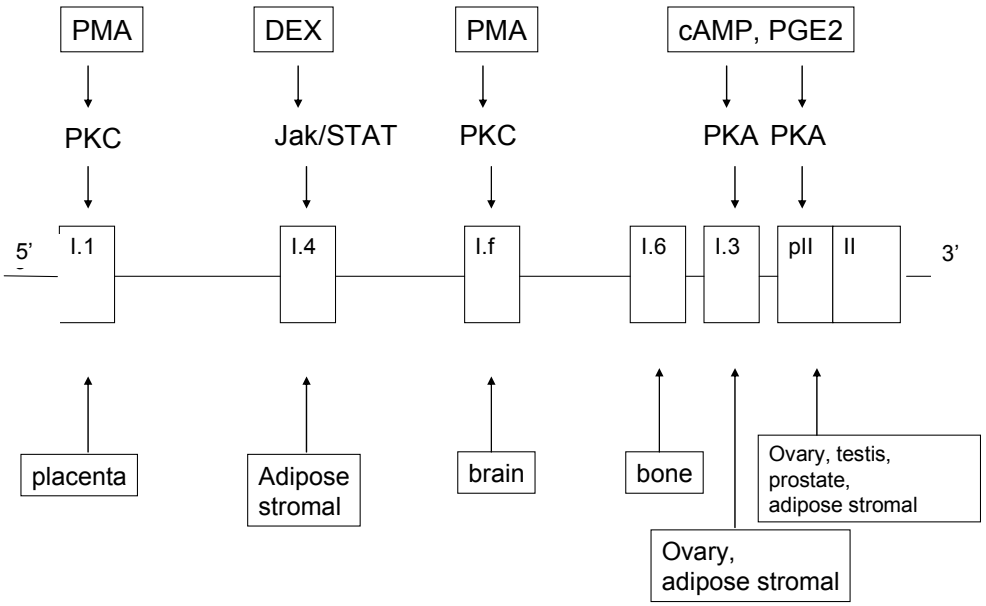


Figure 7: Schematic overview of aromatase promoter region, with exon II being the first coding region for the aromatase protein [1,5].

The H295R cell line originates from human adrenocortical tissue. The major function of the adrenal cortex is to produce a wide range of biologically active steroids and therefore, multiple pathways of steroidogenesis are present. They are primarily involved in the formation of glucocorticoids, mineralocorticoids and weak androgens. Additionally, in H295R cells, small amounts of estrogens are secreted.[24] Androgens are released from the adrenal cortex into the bloodstream and transported to other organs where they can be converted to estrogens by the aromatase enzyme.[44] H295R cells express aromatase and are able to synthesize estrogens. To our knowledge, this is the first study of aromatase regulation in the

H295R cell line. The present study has shown that aromatase activity is increased after a 24h exposure to cAMP, DEX, PGE2 and PMA, indicating that several different promoter regions can be activated in the human adrenocortical carcinoma cell line H295R (figure 7).

We are aware of the fact that presence of serum in cell culture medium can influence the inducibility of aromatase activity by different chemicals. For example, DEX was only able to induce aromatase activity in adipose stromal cells when cultured in medium containing fetal bovine serum. The opposite was true for induction by cAMP and PMA.[1] However, we showed that DEX was not able to induce aromatase activity in R2C cells. Additionally, cAMP and PMA were able to induce aromatase activity in H295R cells even though serum was present.

Depending on the inducer added to the H295R cells, a specific promoter region is activated which increases aromatase gene transcription. This property potentially allows this cell line to be used for screening of chemicals for their ability to induce aromatase activity, and to identify the mechanism of induction. Exposure of H295R cells to 30 μM ATR resulted in a 3-fold induction of aromatase activity, which was shown to correlate with increased intracellular cAMP levels.[12] Exposure of H295R cells to 30 μM of ATR, resulted in a 175% increase in cAMP levels and a 250 % increase in aromatase activity, whereas 20 μM of forskolin resulted in a 400 % increase in cAMP levels and similar 4 fold increase in aromatase activity;[12] thus ATR is a less efficacious aromatase inducer than forskolin.

Aromatase enzyme activity was increased in H295R cells after exposure to 8-Br-cAMP, PMA, PGE2, DEX and ATR. After the same treatment, amplification responses of pII specific- as well as I.3 specific transcripts were elevated. We were not able to detect a I.4 specific transcript. When using the same I.4 specific primers in primary human mammary fibroblasts, a clear and intense PCR product was obtained, indicating that the primers were able to recognize the I.4 promoter region. Since H295R is a cell line and promoter I.4 is located approximately 70 kb from the translation initiation site, it is possible that this specific promoter region is altered and hence not recognized by our primers. However, H295R exposure to DEX did result in

increased aromatase enzyme activity. I.3 and pII specific transcript amplification response was not significantly elevated, indicating that increase of the enzyme activity might have taken place via the I.4 promoter region.

Aromatase regulation and cancer

Approximately 60% of all breast tumors are estrogen-responsive.[45] Promoter regions of the aromatase gene play a role in the regulation of aromatase expression in various tissues such as brain, placenta, healthy and diseased gonadal and breast tissue. Subsequently, they may also play an important role in tumor genesis, development, behavior and reproduction. It is postulated that in healthy breast tissue estrogen production by aromatase in tumor surrounding fibroblasts is mainly regulated by the I.4 promoter region of the aromatase gene, which is glucocorticoid responsive. However, in breast carcinoma cases, aromatase activity and thus local estrogen production is increased and is regulated mainly by promoter regions I.3 and pII, which are cAMP responsive.[46-48] Switching of promoter regions is an important etiological factor in the development of breast carcinoma. If xenobiotics can activate a specific promoter region of the aromatase gene or can modulate the promoter-switching event, such compounds can in turn induce aromatase activity and estrogen production.

Conclusions

H295R cells were shown to be able to detect induction as well as inhibition of aromatase activity. Since ATR is known to enhance aromatase activity via the PKA phosphorylation pathway and had a tendency to increase transcription of aromatase promoter regions I.3 and pII, the H295R cell line appears to be useful for detection of aromatase inducers as well as inhibitors. The R2C cell line, although not suitable for studying induction, appears to be a more sensitive cell line than H295R for studying inhibitory effects of xenobiotics on aromatase activity. We conclude that the H295R cell line contains aromatase promoter regions, which are responsive to stimulants of various second messenger pathways, such as PKA, PKC and Jak/STAT. This feature may make the H295R cell line a useful tool for endocrine disruptor screening purposes.

Acknowledgements

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Inhibition of aromatase activity by methyl sulfonyl PCB metabolites in primary culture of human mammary fibroblasts

4

Marjoke Heneweer[1], Martin van den Berg[1], Maarten C. de Geest[1], Paul C. de Jong[2], Å. Bergman[3] and J. Thomas Sanderson[1]

[1] Institute for Risk Assessment Sciences (IRAS), Utrecht University, PO Box 80176, 3508 TD Utrecht, The Netherlands.

[2] St. Antonius Hospital, PO Box 2500, 3430 EM Nieuwegein, The Netherlands.

[3] Department of Environmental Chemistry and Analytical Chemistry, Stockholm University, SE-10691 Stockholm, Sweden.

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Abstract

In this study, effects on catalytic activity and mRNA levels of aromatase in primary human mammary fibroblasts were evaluated after exposure to promoter-specific modulators of aromatase expression and methyl sulfonyl polychlorinated biphenyl metabolites ($MeSO_2$-PCBs). A method for fibroblast isolation from primary breast tissue was developed and optimized, and aromatase activity and promoter-specific mRNA levels were assessed in these cells after exposure to test compounds. A 24h exposure of fibroblasts to dexamethasone (DEX) (1-100 nM) increased aromatase activity to a maximum of 313-fold. DEX also elevated promoter I.4-specific RNA levels. A 24h exposure of fibroblasts to 3-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-91, or 4-$MeSO_2$-PCB-149 (0.1 – 10 µM) resulted in concentration-dependent decrease of aromatase activity. Exposure of fibroblasts to $MeSO_2$-PCBs just for the limited duration (6h) of the catalytic assay, caused a concentration-dependent inhibition of aromatase enzyme activity. mRNA levels were not altered by a 24h $MeSO_2$-PCB exposure nor was cytotoxicity observed. In aromatase expressing human adrenocortical carcinoma H295R cells, a 24h exposure to 3-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-91, or 4-$MeSO_2$-PCB-149 (0.1 – 10 µM) also resulted in a concentration-dependent decrease of aromatase activity. Additionally, there were no changes in aromatase mRNA levels after 24h exposure of H295R cells to $MeSO_2$-PCBs. We conclude that in primary human mammary fibroblasts as well as in H295R cells, aromatase inhibition by $MeSO_2$-PCBs is likely to be due to catalytic inhibition.

Introduction

Since approximately 60% of all breast tumors are estrogen-responsive,[1,2] compounds that show estrogenic or anti-estrogenic properties are able to influence breast tumor growth. Epithelial breast tumor cells express estrogen receptors to which endogenous and/or exogenous estrogens can bind. In a breast tumor, adipose stromal cells surrounding the epithelial tumor cells contain the aromatase enzyme, which is responsible for converting androgens into estrogens.[3] Exposure to compounds that induce aromatase activity in the breast can therefore lead to increased estrogen levels and possibly to accelerated breast tumor growth. Through this mechanism, a positive feedback loop can be established in which factors secreted from epithelial tumor cells stimulate aromatase activity in surrounding fibroblasts. Subsequently, products (synthesized estrogens) of the fibroblasts enhance epithelial tumor cell growth.[4] Therefore, it is important to identify exogenous compounds that can alter aromatase activity in addition to those compounds which have direct interaction with the estrogen receptor (ER).

Aromatase (CYP19) comprises the ubiquitous flavoprotein, NADPH-cytochrome P450 reductase, and a unique cytochrome P450 that is exclusively expressed in estrogen producing cells.[5] Previous studies have revealed that expression of the aromatase gene is regulated in a species- and tissue specific manner.[2,3,6] In healthy breast tissue, the predominantly active aromatase promoter region I.4 is regulated by glucocorticoids and class I cytokines.[5] In breast tumor tissue, cyclic adenosine monophosphate (cAMP) and prostaglandin E2 (PGE2) levels are elevated and the protein kinase A (PKA) phosphorylation pathway-responsive aromatase promoter regions pII and I.3 become activated. This promoter-switching event from I.4 to I.3/pII results from changes in the presence of mediators rather than intrinsic differences in fibroblasts.[4,7] Promoter II (pII)-specific expression of aromatase in adipose stromal cells is induced by PGE2 and further potentiated by phorbol esters, suggesting that both PKA and protein kinase C (PKC) pathways are involved in optimal expression, although phorbol esters themselves have no stimulatory activity.[8] Furthermore, aromatase gene expression via promoters II and

I.3 is also elevated after exposure to the widely used herbicide atrazine in human adrenocortical carcinoma H295R cells.[9]

The current paper describes effects on catalytic aromatase activity and promoter I.4-specific mRNA levels after exposure of primary human fibroblasts to promoter specific modulators of aromatase activity as well as methyl sulfonyl polychlorinated biphenyl metabolites ($MeSO_2$-PCBs). $MeSO_2$-PCBs are persistent contaminants and are ubiquitously present in humans and the environment. Lipophilicity of $MeSO_2$- PCB metabolites is similar to the parent compounds and they have been detected in human milk, adipose, liver and lung tissue.[10] 4- $MeSO_2$-PCB-149 is the most abundant PCB metabolite in human adipose tissue and milk at a level of 1.5 ng/g lipids.[11] Human blood concentration of 4-$MeSO_2$-PCB-149 is approximately 0.03 nM.[11,12] 3-$MeSO_2$-PCB-101 is the predominant PCB metabolite in muscle and blubber in wildlife, such as otter, mink and grey seal.[11] In the environment, they have been linked to chronic and reproductive toxicity in exposed mink.[13] Additionaly, some $MeSO_2$-PCBs have been shown to be glucocorticoid receptor (GR) antagonists.[14] Therefore, it is important to investigate possible aromatase inhibiting properties of $MeSO_2$-PCBs in relevant human tissues. We chose to use primary human mammary fibroblasts because of their role in breast cancer development. We compared the results in primary fibroblasts with results that we obtained when using the human adrenocorticocarcinoma cell line H295R. Previous studies proved these cells to be a suitable tool for studying inhibitory effects of xenobiotics on aromatase activity.[9]

The aims of this study were to establish an efficient method for isolation of fibroblasts from primary breast tissue. Furthermore, we wanted to investigate effects on aromatase by $MeSO_2$-PCB exposure and elucidate the mechanism of action.

Methods

Isolation of fibroblasts from human mammary tissue

Fibroblast isolation was performed according to a method developed by Van de Ven and co-workers [15] with modifications. Mammary tissue was collected after informed consent, from 9 different women undergoing reduction mammoplasty in the St. Antonius Hospital in Nieuwegein, The Netherlands. Average age was 32.3 years, ranging from 17-52 years. Tissue collection was approved by the Medical Ethical Committee (TME/Z-02.09) of the St. Antonius Hospital. After pathological examination, the tissue was classified as healthy. After surgery, tissue was transported to the laboratory as quickly as possible in sterile PBS buffer on ice. Upon arrival, tissue was placed in 70% EtOH for 4 seconds and washed twice with sterile PBS. Subsequently, tissue was minced in a sterile glass petridish using sterile scalpels and scissors and transferred to a sterile Erlenmeyer flask. Sterile Krebs Ringer buffer (18 mM NaCl, 5.4 mM KCl, 1.6 mM NaH_2PO_4, 21.9 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11.1 mM glucose, equilibrated with carbogen, pH = 7.4) containing collagenase I (150U/ml) was filtered through a 0.2 μm filter and added to the minced tissue. Tissue digestion was performed in shaking waterbath at 37°C for 2.5h. After digestion, tissue was sieved and centrifuged at 1000g for 20 min. The obtained pellet contained the fibroblasts. Fat and buffer were removed with a pipet and warm culture medium (RPMI 1640, 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1 μg/ml insulin) was added to the cells. Cells were plated in 25 cm^2 tissue culture flasks and kept at 37°C and 5% CO_2. Medium was changed for the first time after 2 days to remove fat and blood and once a week thereafter.

Cell culture and exposure

Primary human mammary fibroblasts were plated in 6 well plates at a density of approximately $1x10^5$ cells/well. After 24h, culture medium was replaced with 3 ml of fresh culture medium and 3 μl of the test compound was added to the wells.
Phorbol 12-myristate 13-acetate (PMA), prostaglandin E2 (PGE2), dexamethasone

(DEX) and 8-bromo-cyclic AMP (8-Br-cAMP) were obtained from Sigma Aldrich (Sigma-Aldrich, Zwijndrecht, The Netherlands). 8-Br-cAMP and PMA were dissolved in DMSO (Sigma Aldrich, Zwijndrecht, The Netherlands). Dexamethasone and PGE2 were dissolved in 70% ethanol. All exposures were performed at 37°C, 5% CO_2 for 24h.

The human adrenocorticocarcinoma cell line H295R [16] was obtained from the American Type Culture Collection (ATCC # CRL-2128) and grown in 1:1 Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12) containing 365 mg/ml L-glutamine and 15 mM HEPES (GibcoBRL 31300-038). The culture medium was supplemented with 10 mg/l insulin, 6.7 μg/l sodium selenite and 5.5 mg/l transferrin (ITS-G, GibcoBRL 41400-045), 1.25 mg/ml bovine serum albumin (Sigma A9647), 100 U/l penicillin/100 μg/l streptomycin (GibcoBRL 15140-114) and 2% steroid-free replacement serum Ultroser SF (Biosepra, France).

^{3}H-water release assay for aromatase activity

Catalytic activity of aromatase was determined by performing a ^{3}H-water release assay, after Lephart and Simpson [17] with modifications.[18] The activity of aromatase was corrected for the distribution of the ^{3}H-label on the androstenedione molecule, which was 25.7% at the 1α position and 74.2 % at the 1ß-position.[19] Briefly, serum-free medium, containing 20 nM 1β-^{3}H-androstenedione (New England Nuclear Research Products, Boston, MA, USA) was added to the fibroblasts after 24h exposure to the test compound. After a 6h incubation at 37°C, 5% CO_2, the reaction was stopped by putting the cells on ice. 800 μl of medium was extracted with 2000 μl $CHCl_3$ and vortexed for 15 s. A centrifugation step at 11000*g* for 2 minutes was included before transferring 400 μl of aqueous phase to a vial containing 400 μl dextran coated charcoal to remove the last organic compounds. The suspension was vortexed for 15s and incubated for 5 minutes. Subsequently, the samples were centrifuged at 11000*g* for 15 min and 500 μl of aqueous phase was transferred to a scintillation vial, to which 4 ml of scintillation cocktail (Perkin Elmer, Boston, MA, USA) was added. Finally, radioactivity was counted in liquid

scintillation counter. The specificity of this radiometric assay in primary human fibroblasts was verified by using 4-hydroxyandrostenedione, a specific irreversible inhibitor of the catalytic activity of aromatase.[18,20]
An assay based on the method of Lowry [21,22] for protein determination was performed to correct for differences in cell numbers between the wells.

mRNA levels

RNA was isolated from fibroblasts using the RNA Instapure System (Eurogentec, Liège, Belgium) according to the enclosed instructions. Purity of RNA was assessed by measuring 260/280 nm wavelength ratio. RNA was stored in –70°C at a concentration of 50 ng/µl. For primary human mammary fibroblasts, specific aromatase promoter region I.4, primers designed by Bouraïma [9,23] were used. For H295R cells, we used primers for exon II.[24] Primers for β-actin served as internal control.[9] Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the Access RT-PCR System (Promega, Madison, WI, USA) according to the supplied protocol. For promoter region I.4, annealing temperature was 60°C, extension lasted 1 minute and 30 cycles were performed. $MgSO_4$-concentration was 1mM. For exon II of aromatase, annealing temperature was 57°C, extension lasted 2 minutes and 40 cycles were performed. $MgSO_4$-concentration was 0.75 mM. Amplification product of 475 bp for promoter region I.4 and 318 bp for exon II was detected using 2% agarose gel electrophoresis and ethidium bromide staining. Intensity of the stains was quantified using a FluorImager (Molecular Dynamics, USA). Previous experiments (data not shown) showed that RT-PCR was performed under conditions of linearity, in order to obtain semi-quantitative amplification responses.

Test for cell viability

Mitochondrial function as an indicator for cell viability, was assessed by measuring the capacity of human primary fibroblasts to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan [25] after 24h exposure to test compound. The cells were plated on a 24-wells plate and incubated

for 30 min, at 37°C with 0.5 ml of MTT (1 mg/ml), dissolved in serum-free medium. MTT solution was removed and cells were washed twice with PBS. Formazan that was formed in the cells was extracted with 1 ml isopropanol. Absorbance was measured spectrophotometrically (Fluostar, BMG Labtechnologies, Offenburg, Germany) at a wavelength of 595 nm.

Data analysis

Human tissue samples were not pooled and each sample served as its own control. For graphs and plotting of dose-response curves, Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA) was used. All error bars are presented as standard error of the mean (S.E.M.). To identify statistically significant differences among means, a one-way ANOVA followed by Dunnett's posteriori-test were performed.

Results

Promoter specific stimulants

Treatment of primary human fibroblasts with several stimulants of different aromatase promoter regions, resulted in a 3-, 634-, and 54-fold increase in aromatase activity for 100 µM 8-Br-cAMP, 100 nM DEX and 1µM PGE2, respectively (figure 1).

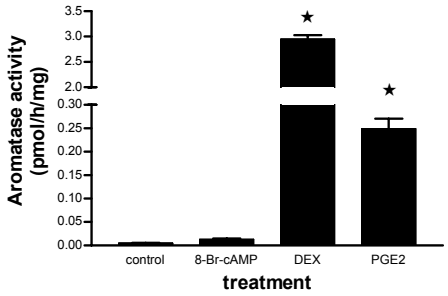


Figure 1: Aromatase activity in primary human fibroblasts after 24h exposure to promoter-specific stimulants: 100 µM 8-Br-cAMP, 100 nM dexamethasone and 1 µM PGE2. Error bars are standard errors. Each bar is average of triplicate measurements (n=3). Each experiment was performed in 3 different fibroblast samples. Graph shows one representative experiment. (*) Significantly different from control ($p < 0.002$).

In healthy human breast tissue, the glucocorticoid-mediated mechanism of aromatase induction is most relevant. Therefore, we decided to further investigate this mechanism. A 24h exposure of primary human fibroblasts to different concentrations (1-100 nM) of DEX resulted in a concentration-dependent increase in aromatase activity (figure 2). Maximum increase of aromatase activity that was observed, was 313-fold at concentrations around 100 nM. It should be noted that tissue obtained from different patients, showed very large differences in basal aromatase activity and inducibility. Basal aromatase activity ranged from 0.005 to 0.09 pmol/h/mg protein (n=9). After induction by 100 nM DEX for 24h, activities ranged from 0.2 to 5.8 pmol/h/mg protein. There was no correlation between basal activity and inducibility of aromatase (figure 3). Induction ranged from 7- to 634-fold.

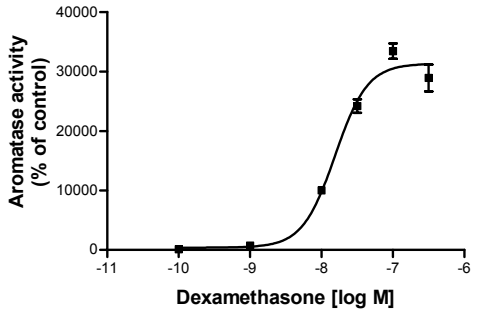


Figure 2: Aromatase activity (% of control) in primary human fibroblasts after 24h exposure to different concentrations of dexamethasone. Error bars represent standard errors. Each point is average of triplicate measurements (n=3). Each experiment was performed in 3 different fibroblast samples. Graph shows one representative experiment.

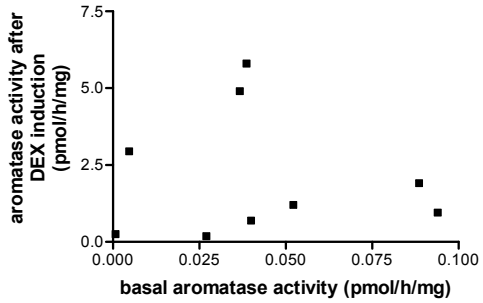


Figure 3: Inter-individual differences in basal aromatase activity and inducibility (100 nM dexamethasone for 24h) in primary human fibroblasts.

In order to check for the presence of a glucocorticoid dependent pathway of aromatase induction, aromatase promoter I.4-specific RNA levels were quantified after exposure to DEX. A 24h exposure to 100 nM DEX resulted in a 1.7-fold increase in aromatase promoter I.4-specific RNA levels compared with solvent control (figure 4).

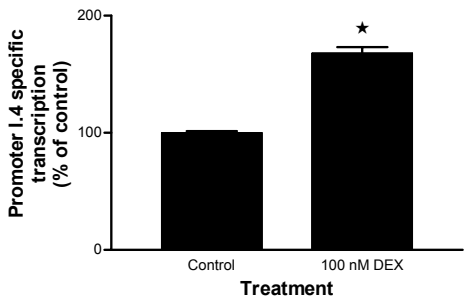


Figure 4: Promoter I.4 specific mRNA transcripts in primary human fibroblasts after 24h exposure to solvent control or 100 nM dexamethasone (DEX). Error bars represent standard errors. Each point is average of triplicate measurements (n=3). Each experiment was performed in 3 different fibroblast samples. Graph shows one representative experiment. (*) Significantly different from control (p = 0.0002).

Inhibition of aromatase activity by $MeSO_2$-PCB metabolites

Exposure of primary human mammary fibroblasts for 24h to 10 μM of 3-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-149, or 4-$MeSO_2$-PCB–91 each resulted in concentration-dependent decrease of aromatase activity (from 100% to approximately 20%) (figure 5, left panel). For 3-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-132, and 4-$MeSO_2$-PCB-149, estimated IC_{50} values were < 1 μM. For 4-$MeSO_2$-PCB–91, the estimated IC_{50} value was between 1 and 3 μM. Cytotoxicity was not observed at concentrations up to 10 μM (data not shown). When primary fibroblasts were exposed to different concentrations of the $MeSO_2$-PCBs just for the limited duration of the assay (6 hours), aromatase enzyme activity decreased concentration dependently from 100% to 20% (figure 5, right panel).

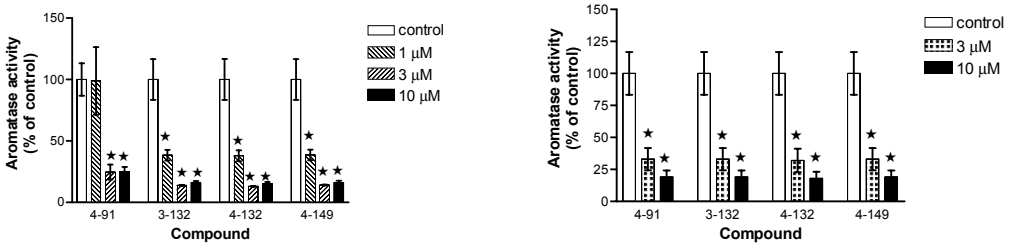


Figure 5: Left: Aromatase activity in primary human fibroblasts after 24h exposure to 0, 1, 3 or 10 μM of 3-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -149, 4-$MeSO_2$-PCB -91. (*) Significantly different from control ($p < 0.01$, except for 4-91: $p < 0.05$). Right: Aromatase activity in primary human fibroblasts after 6h exposure to 0, 3 or 10 μM of 3-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -149, 4-$MeSO_2$-PCB -91. (*) Significantly different from control ($p < 0.05$). Left and right graphs are constructed from 2 different fibroblast samples. Absolute basal aromatase activities were 0.008 (3-132, 4-132 and 4-149) and 0.02 (4-91) pmol/h/mg protein. Error bars are standard errors. Bars represent average of triplicate measurements (n=3). Each experiment was performed in 3 different fibroblast samples. Graphs show one representative experiment.

To check whether aromatase inhibition by $MeSO_2$-PCBs was (partly) caused by down regulation of gene expression, fibroblasts were exposed to the synthetic GR-antagonist RU486 as a positive control, as well as to $MeSO_2$-PCBs. Enzyme activity as well as promoter I.4-specific mRNA levels were quantified after 24h. When fibroblasts were co-exposed to the EC_{50} value of DEX and different concentrations of the synthetic anti-glucocorticoid RU486, aromatase enzyme activity was completely inhibited at a concentration of 100 nM, with an estimated IC_{50} value of 10 nM (figure 6).

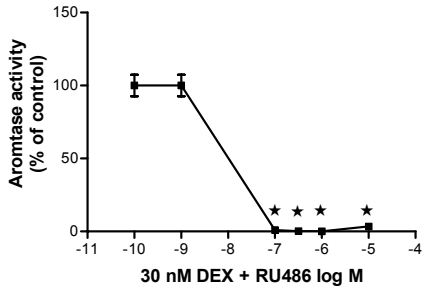


Figure 6: Aromatase activity in primary human fibroblasts after 24 h exposure to dexamethasone (EC_{50} value) and different concentrations of the synthetic glucocorticoid receptor antagonist RU486. Error bars are standard errors. Each point is average of triplicate measurements (n=3). Each experiment was repeated in 3 different fibroblast samples. Graph shows one representative experiment. * Significantly different from control ($P < 0.05$).

When fibroblasts were exposed to 30 nM (EC_{50} value) DEX and RU486, promoter I.4-specific RNA levels dropped to 13% of control value at a RU486 concentration of 100 nM (figure 7, upper panel). However, exposure of the primary human fibroblasts to solvent control or 10 μM of 3-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-132, 4-$MeSO_2$-PCB-149, or 4-$MeSO_2$-PCB–91, did not alter levels of aromatase promoter I.4-specific RNA transcripts, which were elevated by simultanous exposure to DEX at EC_{50} value (figure 7, lower panel).

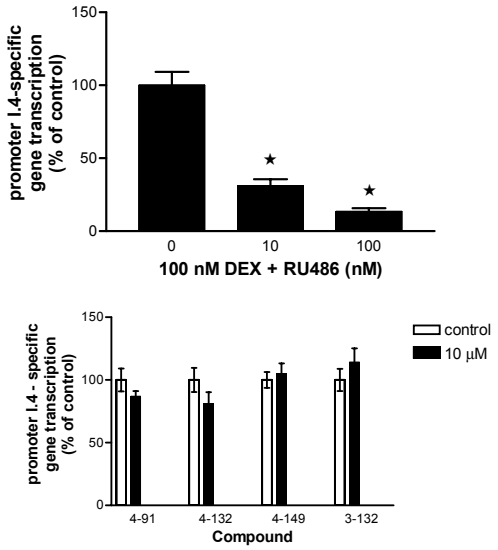


Figure 7: Upper: Promoter I.4 specific mRNA transcripts in primary human fibroblasts after 24h exposure to dexamethasone (EC_{50} value) and different concentrations of the synthetic glucocorticoid receptor antagonist RU486. Error bars are standard errors. Bars represent average of triplicate measurements (n=3). Each experiment was repeated in 2 different fibroblast samples. Graph shows one experiment. (*) Significantly different from control ($p < 0.05$). Lower: Aromatase promoter I.4-specific gene transcription in primary human fibroblasts after 24h exposure to EC_{50} of dexamethasone and 0 or 10 μM of 3-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -149, or 4-$MeSO_2$-PCB -91. Error bars are standard errors. Bars represent average of triplicate measurements (n=3). Each experiment was repeated in 3 different fibroblast samples. Graph shows one representative experiment.

Effects of $MeSO_2$-PCB metabolites on aromatase activity in H295R cells

DEX-treated H295R cells were exposed to different concentrations of 3-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB –149 or 4-$MeSO_2$-PCB –91, which resulted in concentration-dependent inhibition of aromatase activity (figure 8, upper panel). EC_{50} values were 0.9, 2, 1.8 and 0.6 μM, respectively. Cytotoxicity was not observed at concentrations up to 10 μM (data not shown). Exposure of the H295R cells to solvent control or 10 μM of 4-$MeSO_2$-PCB–91, did not alter levels of aromatase transcripts. However, exposure to 10 μM of 4-$MeSO_2$-PCB-132 resulted in a slight, but statistically significant ($p<0.05$) decrease in aromatase transcripts compared to solvent control (figure 8, lower panel).

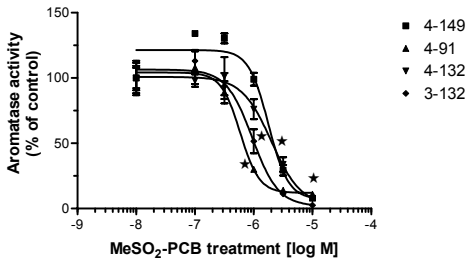
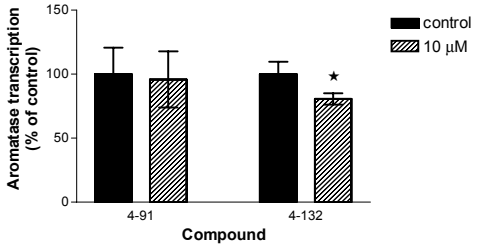


Figure 8: Upper: Aromatase activity in DEX-treated H295R cells after 24h exposure to different concentrations of 3-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -132, 4-$MeSO_2$-PCB -149, 4-$MeSO_2$-PCB -91. Error bars are standard errors. Points represent averages of triplicate measurements (n=3). Each experiment was repeated in 3 different fibroblast samples. Graph shows 1 representative experiment. (*) Significantly different from control ($p < 0.01$).



Lower: Aromatase gene transcription in primary human fibroblasts after 24h exposure to 0 or 10 μM of 4-$MeSO_2$-PCB-91 or 4-$MeSO_2$-PCB-132. Error bars are standard deviations. Bars represent average of triplicate measurements (n=3). Each experiment was repeated in 2 different fibroblast samples. Graph shows one experiment. (*) Significantly different from control ($p<0.05$).

Discussion

Promoter specific stimulants

The large differences in inducibility of aromatase activity after exposure of primary human mammary fibroblasts to 100 nM DEX, confirm observations by Santner and co-workers, showing that enzyme activity in stromal cells from breast tumor tissue was low basally, but increased by 30- to 1200- fold when induced by DEX.[26] Catalytic aromatase enzyme activity and mRNA levels were affected by RU486 treatment, suggesting the presence of a glucocorticoid-mediated mechanism of induction and inhibition of aromatase enzyme activity. Our results with RU486 confirm results described by Schmidt and Loffler.[27]

Inhibition of aromatase activity by $MeSO_2$-PCB metabolites

A decrease in enzyme activity can be caused by direct catalytic inhibition of the enzyme and/or down regulation of gene expression. To test for catalytic inhibition, primary human fibroblasts were exposed to $MeSO_2$-PCBs for the duration of the aromatase assay only and a decrease of aromatase activity was observed. Because exposure of primary human fibroblasts was only for 6h, and no effects on the RNA level were observed after 24h exposure, little to no effect on RNA synthesis is likely to have taken place. This indicates a direct effect of the $MeSO_2$- PCB metabolites on the aromatase enzyme. A large variability between interindividual fibroblast samples was observed. Absolute basal aromatase activities ranged from 0.005 to 0.09 pmol/h/mg protein (fig. 3). However, in spite of these differences, inhibitory effects on aromatase activity of $MeSO_2$- PCB metabolites were similar and reproducible among different fibroblast samples when control values were set to 100%. Letcher showed a decrease of aromatase activity in JEG-3 and JAR cells after exposure to several organochlorines. However, this was due to cytotoxicity.[28] This was not the case in primary human fibroblasts.

Effects of $MeSO_2$-PCB metabolites on promoter I.4-specific mRNA levels

Some $MeSO_2$-PCBs have affinity for the GR and are able to interfere with the glucocorticoid signaling pathway.[14] Therefore, we expected the $MeSO_2$-PCBs to inhibit aromatase activity in primary human fibroblasts due to their antiglucocorticoid properties and subsequent ability to downregulate promoter I.4 regulated aromatase activity. Under our experimental conditions, basal mRNA levels were around detection limit. That is the reason why, as an alternative approach, we decided to induce aromatase gene expression with DEX at EC_{50} before exposing to methyl sulfonyl PCB metabolites. We chose EC_{50} because at this concentration changes in mRNA levels are easiest to be picked up, due to the steep slope of concentration-response curve.

The disparity in levels of induction at the level of the I.4 promoter (2-fold) and the level of aromatase activity (>300 fold) seems large. The 300-fold induction of aromatase enzyme activity, however, was seen in a batch of fibroblasts that had low basal activity by were highly inducible by 100 nM DEX. The 2-fold induction of aromatase RNA levels was not observed in the same batch of fibroblasts. Differences in inducibility of aromatase enzyme activity ranged from 7- to 634-fold between different batches of cells obtained from different individual patients. Furthermore, a semi-quantitative RT-PCR was performed, only allowing for a conclusion whether or not there were changes in RNA levels. Finally, several studies suggest that glucocorticoids do interfere with mRNA stability of other genes.[29,30] However, there are no indications in literature that glucocorticoids post-transcriptionally influence aromatase mRNA stability.

The concentration-dependent decrease of aromatase promoter I.4-specific mRNA levels after exposure to the EC_{50} of DEX and different concentrations of RU486, indicates that decrease of aromatase activity through downregulation of CYP19 mRNA is possible in these cells. However, exposure of fibroblasts to the EC_{50} of DEX and different concentrations of $MeSO_2$-PCBs, did not result in any significant changes in aromatase promoter I.4-specific mRNA levels, indicating that interaction

with the GR does not play a role in aromatase inhibition by $MeSO_2$-PCBs in primary human mammary fibroblasts.

Comparison with H295R cells

The human adrenocorticocarcinoma cell line H295R has been shown to be a suitable tool for studying inhibitory effects on aromatase activity.[9,24] Therefore, we compared the results obtained in primary human mammary fibroblasts with those obtained in H295R cells. 100 nM DEX also increased aromatase activity in H295R cells, although to a lesser extent than in primary fibroblasts. IC_{50} values for aromatase inhibition were comparable in H295R cells and primary fibroblasts.

In contradiction to the primary fibroblasts, aromatase mRNA levels were slightly, but significantly altered in H295R only after exposure to 4-$MeSO_2$-PCB-132. We consider this change marginal in comparison with the overwhelming inhibition of aromatase enzyme activity after exposure of the H295R cells to methylsulfonyl PCB metabolites. It must be noted that in H295R cells, primers that were designed for the coding region of the aromatase gene were used.

The lack of biologically significant decreases of aromatase mRNA levels after 24h exposure to $MeSO_2$-PCBs, indicates a direct effect on the aromatase enzyme in H295R, as was already found in primary fibroblasts.

Environmental and biological relevance

Exposure to organochlorines have been linked to possible adverse endocrine effects observed in wildlife, laboratory animals and humans.[31] Numerous effects of PCBs have been demonstrated in experimental animals, some of which can be induced at tissue concentrations not far from body burdens that are present in background exposure population in industrialized countries. Effects at low doses were observed in experiments involving perinatal exposure. For example, severely depressed thyroxine levels (by 60-90%) were observed in fetal and neonatal rats following *in utero* exposure to low doses of Aroclor 1254.[32] Much higher doses of Aroclor 1254 were needed in adult rats to cause similar changes in plasma thyroxine

levels.[33] Perinatal exposure to PCBs did not cause permanent alterations in thyroid hormone levels in the offspring.[34]

Tanabe [35] showed that contamination by organochlorines in marine mammals has reached a level that can cause enzyme induction and endocrine disruption. However, a hazard assessment for fish-eating Mink (*Mustela vison*) based on PCB concentrations that were found in fish [36] led to the conclusion that the contamination levels of PCBs are likely to be safe for the mink.

Human exposure to PCBs has been associated with testicular cancer and decreased sperm counts in males and may play a role in the development of breast cancer in women.[37-39] In human infants from accidentally exposed populations, developmental effects were caused by prenatal exposure to PCBs and included retarded growth, neurobehavioral effects, immune effects, chloracne, and teeth and nail deformities. In studies involving human infants at background environmental exposure levels, subtle changes were observed in thyroid hormone levels and neurobehavioral parameters, associated mainly, but probably not exclusively, with prenatal exposure to PCBs.

The pharmacological agent letrozole, decreases aromatase activity with 80%.[40] To obtain a similar effect on aromatase activity, a $MeSO_2$-PCB-exposure of approximately 10 μM is needed. In human blood, 0.03 nM 4-$MeSO_2$-PCB-149 was detected.[11,12] This means that there is a difference of 5 orders of magnitude between human body burden and pharmacologically active concentrations. Therefore, a biologically significant effect of $MeSO_2$-PCB exposure on aromatase activity is not expected. Looking at $MeSO_2$-metabolites of different PCB congeners, quite large differences in affinity for the GR are observed (table 1). Johansson showed a weak GR-binding at $MeSO_2$-PCB concentrations of approximately 10 μM.[14] Our studies indicated cytotoxicity at concentrations larger than 10 μM. The IC_{50} values for aromatase inhibition in both fibroblasts and H295R cells are lower than the IC_{25} for GR-binding. This confirms our observations that aromatase activity can be inhibited without GR-binding and subsequent decrease of aromatase mRNA synthesis. Furthermore, an increase in affinity for the GR was not reflected in an increase in

aromatase inhibition, suggesting that receptor binding does not always imply biologically significant agonism or antagonism resulting in physiological changes.

A 24h exposure to methylsulfonyl PCB metabolites causes inhibition of aromatase enzyme activity in primary human mammary fibroblasts as well as in H295R cells. However, aromatase RNA levels remain unchanged after 24h exposure to methylsulfonyl PCB metabolites. This observation leads to our overall conclusion that catalytic inhibition is a likely cause for the observed aromatase enzyme inhibition in both primary human mammary fibroblasts and H295R cells.

Table 1: overview of glucocorticoid receptor (GR)-binding and aromatase inhibitory properties of methyl sulfonyl polychlorinated biphenyl metabolites

congener	**GR binding** IC_{25} (μM) (Johansson *et al.*, 1998)	**aromatase inhibition** in fibroblasts estimated IC_{50} (μM)	**aromatase inhibition** in H295R after DEX exposure IC_{50} (μM)
RU486		0.01	
4-149	5±4	0.9	1.8
4-91	7±4	2.3	0.6
3-174	7.5		--
4-132	9±5	0.9	2
3-91	12		--
3-132	17	0.9	0.9
3-101	--		--

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Co-culture of primary human mammary fibroblasts and MCF-7 cells as an *in vitro* breast cancer model

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Marjoke Heneweer[1], Martine Muusse[1], Milou Dingemans[1], Paul C. de Jong[2], Martin van den Berg[1], J. Thomas Sanderson[1]

[1]*Institute for Risk Assessment Sciences (IRAS), Utrecht University, PO Box 80176, 3508 TD Utrecht, The Netherlands*

[2]*St. Antonius Hospital, PO Box 2500, 3430 EM Nieuwegein, The Netherlands*

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Abstract

Approximately 60% of all breast tumors are estrogen-responsive and chemicals that show estrogenic or anti-estrogenic properties are able to interact with breast tumor growth. In a breast tumor, adipose stromal cells (fibroblasts) surrounding the epithelial tumor contain the aromatase enzyme, which converts androgens into estrogens. Exposure to aromatase inducers can therefore lead to increased estrogen levels and possibly to accelerated breast tumor growth. Subsequently, breast tumor cells synthesize and secrete elevated levels of factors such as prostaglandin E2 (PGE2), interleukin-6 (IL-6) and IL-6 soluble receptor (IL-6sR), which in turn have the ability to stimulate aromatase gene transcription in fibroblasts, establishing a positive feedback loop. In this study, a technique that allows for culturing MCF-7 epithelial breast tumor cells and healthy primary human mammary fibroblasts together was developed. First attempts aimed to grow the two cell types in two compartments, seperated by a sterile mesh. In this set-up, MCF-7 cell proliferation and aromatase activity in fibroblasts were chosen as endpoints. However, paracrine interactions between the cell types were not established, probably due to dilution of the secreted factors or the absence of direct cell-cell contact. Subsequently, MCF-7 cells and fibroblasts were seeded directly on top of eachother. pS2 and aromatase gene transcription were chosen as endpoints in this one-compartment set-up. To establish the positive feedback loop, the co-culture was exposed to estrogenic compounds. RNA was isolated and reverse-transcriptase polymerase chain reaction (RT-PCR) was performed. Exposure of the co-culture to estradiol (E2), diethylstilbestrol (DES) and bisphenol-A (BPA), resulted in a three- to seven-fold increase of pS2 transcription levels. Furthermore, pS2 transcription levels increased even more when the aromatase substrate testosterone (20 nM) was present in the co-culture medium. Exposure of the co-culture to the aromatase inducer dexamethasone (DEX) resulted in increased pS2 transcription levels, as well as increased aromatase transcription levels. Simultaneous exposure to DEX and the synthetic anti-estrogen ICI 182,780 almost completely blocked the pS2 response. The aromatase induction response was not altered by ICI 182,780 treatment. Simultaneous exposure to DEX and the non-steroidal aromatase inhibitor fadrozole, abolished the effect of testosterone in the co-culture medium, but did not result in pS2 gene transcription levels as low as seen after exposure to ICI 182,780. Taken together, these observations indicate the presence of a positive feedback loop in our co-culture system. This co-culture provides a more sophisticated and sensitive system to detect direct and indirect estrogenic effects of compounds and their possible effects on breast tumor promotion.

Introduction

Estrogenic effects of chemicals have been subject of extensive research during the past years. MCF-7 cells have been used to perform classical studies on cell proliferation and estrogen receptor (ER) binding in order to study the estrogenic effects of various compounds including environmental contaminants. In the U.S., companies are obliged to test for estrogenic effects of their new compounds before being allowed to market them. In Japan and Europe, legislation forcing companies to perform tests for estrogenicity is being introduced.[1]

Studies on chemicals affecting estrogen synthesis have attracted increasing attention. The aromatase enzyme is responsible for converting androgens into estrogens.[2] Various effects on aromatase activity by flavones,[3] commonly used pesticides,[4-8] imidazole drugs [9] and organochlorines have already been described. By enhancing estrogen synthesis, chemicals can exert an indirect estrogenic effect. For example, the widely used herbicide atrazine does not bind to the ER but is capable of increasing aromatase activity approximately three-fold in the human adrenocortical carcinoma cell line H295R.[6] Such an increase of aromatase activity can theoretically result in higher estrogen levels leading to an indirect estrogenic effect.

Aromatase (CYP19) comprises of the ubiquitous flavoprotein, NADPH-cytochrome P450 reductase, and a unique cytochrome P450, which is exclusively expressed in estrogen producing cells.[10] Previous studies have revealed that expression of the aromatase gene is regulated in a species- and tissue specific manner by use of alternate promoter regions.[11,12]

Since approximately 60% of all breast tumors are estrogen responsive,[13,14] estrogenic chemicals have the potential to stimulate breast tumor growth. In this case, epithelial breast tumor cells express estrogen receptors to which endogenous and/or exogenous estrogens can bind. Estrogens in a breast tumor are formed in the surrounding stromal cells by the aromatase enzyme.

In case of an estrogen-responsive breast tumor, estradiol (E2) that is secreted by the fibroblasts surrounding the tumor can enhance tumor cell growth. In

turn, increased numbers of epithelial tumor cells, secrete increased amounts of cytokines and growth factors,[15] such as interleukin-6 (IL-6),[16] interleukin 6 soluble receptor (IL-6sR), [17,18] and prostaglandin E2 (PGE2) [14] which, once diffused to fibroblasts, can induce aromatase gene expression in those cells. Cell culture and nude mouse experiments using aromatase transfected MCF-7 cells, have demonstrated that aromatase expressed in breast cancer cells can play a role in stimulating the growth of tumors.[19,20] Furthermore, co-culture experiments with aromatase-transfected cells together with untransfected cells, showed that estrogen produced by the transfected cells could also stimulate the growth of untransfected cells in a paracrine manner.[20] Abnormal expression of aromatase in fibroblasts surrounding epithelial cells, especially in postmenopausal women, may have a significant influence on breast tumor maintenance and growth. This leads to manifestation of a positive feedback loop that is capable of accelerating estrogen-responsive tumor growth.

The goal of this study was to set up a co-culture system of MCF-7 cells and healthy primary human fibroblasts in order to establish and study the positive feedback loop *in vitro.* To validate this system, we studied direct and indirect effects of a known aromatase modulator dexamethasone (DEX, synthetic glucocorticoid), the estrogenic compounds estradiol (E2), diethylstilbestrol (DES) and bisphenol A (BPA), the synthetic aromatase inhibitor fadrozole (FAD) and the synthetic anti-estrogen ICI 182,780 on pS2 gene expression in MCF-7 cells and aromatase gene expression in fibroblasts. It was hypothesized that estrogenic compounds directly induce estrogen-related pS2 gene expression in MCF-7 cells. Additionally, estrogenic compounds indirectly induce aromatase expression in primary human fibroblasts, which in the presence of an androgen precursor would result in a synergistic effect on pS2 expression in MCF-7 cells co-cultured with fibroblasts. Furthermore, it was hypothesized that DEX could directly increase aromatase gene transcription and synthesis, subsequently causing an indirect increase in pS2 gene expression levels in the breast tumor cells.

Methods

Fibroblasts isolation

Human mammary fibroblasts were isolated from mammary tissue and cultured according to previously published methods.[21] Tissue was pathologically classified as healthy. Approximately three weeks after isolation, fibroblasts were used to establish a co-culture together with the human mammary carcinoma MCF-7 cells (ATCC# HTB-22).

TWO COMPARTMENT CO-CULTURE

Experimental design

On day 1, fibroblasts were plated on the bottom of six-well plates with sterile inserts (Corning-Costar, Schiphol-Rijk, The Netherlands) in culture medium. Culture medium consisted of RPMI 1640, containing L-glutamine, without phenol red (GibcoBRL 11835-030). The medium was supplemented with 10% heat-inactivated fetal calf serum (FCS), 100U/l penicilline/100 µg/l streptomycin (GibcoBRL 15140-114) and insulin (10 µg/ml). Pore size of the membrane was 0.4 µM. Furthermore, MCF-7 cells were washed with sterile phosphate buffered saline (PBS) and put on steroid-free assay medium, containing dextran-coated charcoal (DCC) treated fetal calf serum (FCS). On day 4, when fibroblasts were confluent, MCF-7 cells were trypsinized and seeded in the insert at a density of $1x10^5$ cells/ml (4 ml/well) (figure 1). On day 5, medium was changed and test compounds were added to the cells. On day 8, medium containing the test compounds was replaced with fresh medium and fresh test compounds.

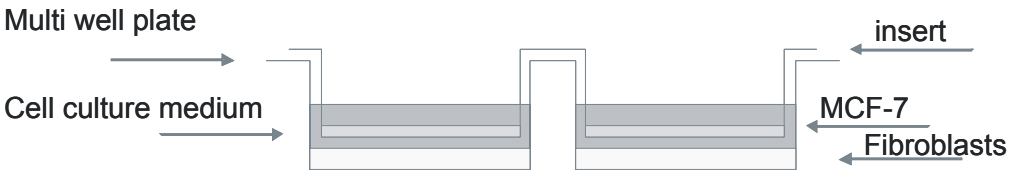


Figure 1: multi-well plates with inserts

Assay for MCF-7 cell proliferation

On day 11 the inserts carrying the MCF-7 cells were transferred to sterile 6-well plates for analysis. Mitochondrial function as an indicator for cell number was assessed by measuring the capacity of MCF-7 cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan .[22] After 72h exposure to test compound, the cells were incubated for 30 min, at 37°C with 2 ml of MTT (1 mg/ml), dissolved in serum-free medium. MTT solution was removed and cells were washed twice with PBS. Formazan that was formed in the cells was extracted with 4 ml isopropanol. Absorbance was measured spectrophotometrically (Fluostar, BMG Labtechnologies, Offenburg, Germany) at a wavelength of 595 nm.

^{3}H-water release assay for aromatase activity

On day 11, a ^{3}H-water release assay was performed on the fibroblasts according to previously published methods.[21]

PGE2 ELISA

Culture medium was sampled on days 5 (before replacement of the medium) and 8. A high-sensitivity prostaglandin E2 kit (Assay designs Inc., Ann Arbor, MI, USA) was used and the ELISA was performed according to enclosed instructions. Briefly, a monoclonal antibody against PGE2 was used to competitively bind PGE2 or an alkaline phosphatase molecule which was covalently bound to PGE2. After simultaneous incubation at 4°C, the excess reagents were washed away and substrate for alkaline phosphatase was added. After incubation at 37 °C, the reaction was stopped and the optical density of the yellow color was measured spectrophotometrically at a wavelength of 405 nm (Fluostar, BMG Labtechnologies, Offenburg, Germany). The intensity of the yellow color was inversely correlated to the concentration of PGE2 in the samples.

IL-6 bioassay

IL-6 dependent 7-TD-1 murine B cell hybridoma cells (kind gift of J. Fink-Gremmels) were cultured in 96-well plates in RPMI 1640 with L-glutamine (Gibco BRL). Culture medium was supplemented with 5% FCS, 1% penicilline/streptomycin

and β-mercapto-ethanol at a concentration of 25 µM. On the day of the assay, all wells were supplied with 50 µl of culture medium.

Co-culture medium was sampled on day 8 and these samples were titrated in threefold dilutions. As a positive control threefold dilutions of recombinant murine IL-6 were used.

Cell proliferation was determined by measuring the increase in mitochondrial activity by performing an MTT-test, as described previously. Experiments were performed in triplo for each sample.

ONE COMPARTMENT CO-CULTURE

Experimental design

On day 1, fibroblasts were plated at a density of approximately 8 x 10^4 cells/well in six-well plates in culture medium. MCF-7 cells were washed with phosphate buffered saline (PBS) and assay medium was added in which heat inactivated FCS was replaced with heat-inactivated, dextran-treated FCS (Hyclone, SH30068.03). On day 4, fibroblasts were washed with PBS and MCF-7 cells were trypsinized and seeded on top of the fibroblasts at a density of 8 x 10^4 cells/well. On day 5, 2 ml of fresh assay medium was added to the co-cultures after which the cells were exposed to 2 µl of test compounds for 72 h (final solvent concentration was 0.1% in medium).

Estradiol (Sigma E2758), dexamethasone (Sigma D1756), ICI 182,780 (Tocris, Bristol, UK) and testosterone (Sigma, T1500) were dissolved in 70% ethanol. Diethylstilbestrol (Sigma D4628) and bisphenol A (Sigma I0635) were dissolved in DMSO (Sigma, D8779). Fadrozole (FAD) was kindly provided by Novartis Pharma AG (Basel, Switzerland) and was dissolved in DMSO. It was used as a non-steroidal positive control for aromatase inhibition.[23,24]

mRNA isolation and quantification

On day 8, RNA was isolated from the co-cultured cells, using the RNA Instapure System (Eurogentec, Liège, Belgium) according to the enclosed instructions. Obtained RNA was a mixture of RNA from primary human mammary

fibroblasts and MCF-7 cells. Purity of RNA was checked by measuring the absorbance ratio at 260/280 nm. RNA was stored at –70°C in aliquots of 50 ng/µl. Primers coding for the estrogen-responsive pS2 gene were used after Lee.[25] A reverse-transcriptase polymerase chain reaction (RT-PCR) (Access, Promega, Madison, WI) was performed with a $MgSO_4$ concentration of 1 mM, annealing temperature of 55 °C and 25 cycles. The primer pair and RT-PCR conditions used for aromatase mRNA amplification was described previously.[6] Amplification products were 189 bp and 314 bp for pS2 and aromatase, respectively, and were detected using agarose denaturing gel electrophoresis and ethidium bromide staining. Intensity of the stains was quantified using a FluorImager (Amersham Bioscience, Piscataway, NJ).

Data analysis

In each experiment, each concentration was tested in triplicate. Each experiment was repeated 2-4 times and found to be reproducible. Each graph shows one representative experiment. Human tissue samples were not pooled, therefore each sample served as its own control. For graphs and statistical analyses, Prism 3.0 (GraphPad Software Inc., San Diego, CA) was used. All error bars are presented as standard error of the mean (SEM). To identify statistically significant differences among means, a one-way ANOVA followed by Tukey-Kramer's posteriori test was performed.

Results

TWO COMPARTMENT CO-CULTURE

Endpoints

To assess effects on aromatase in fibroblasts, we chose to use the ^{3}H-water release assay for aromatase activity. For quantitation of ER-mediated MCF-7 cell proliferation, we chose to use cell viability as an indicator of cell number by performing an MTT-assay. The plating density of MCF-7 cells was optimized by trying 3 different concentrations, resulting in an optimal plating density of $1x10^{5}$ cells/ml (1 ml/well in 24-well plates, data not shown).

Exposure of the co-cultures of primary human mammary fibroblasts and MCF-7 cells to increasing concentrations (1-300 nM) of the synthetic glucocorticoid dexamethasone (DEX), resulted in a concentration-dependent increase of aromatase activity with a maximum effect of 11-fold induction at the highest tested concentration (300 nM). However, no effects on MCF-7 cell proliferation were observed (figure 2A)
When co-cultures were exposed to increasing concentrations of E2 (0.1 pM – 100 nM), MCF-7 cell proliferation increased concentration-dependently up to a concentration of 10 pM. At higher concentrations cell counts dropped drastically. No effects on aromatase activity were observed in the fibroblasts (figure 2B).

PGE2 and IL-6

When co-cultures were exposed to increasing concentrations of E2 (0.1 pM – 10 nM), no increase was observed on PGE2 (figure 3A) or IL-6 concentrations (figure 3B) in the culture medium. In contrast to our expectations, PGE2 levels decreased with increasing E2-concentrations. However, concentrations were higher at day 8 than at day 5. PGE2 concentrations varied around 0.1 pM. IL-6 levels varied around 2000 U/ml and did not increase significantly after E2 exposure.

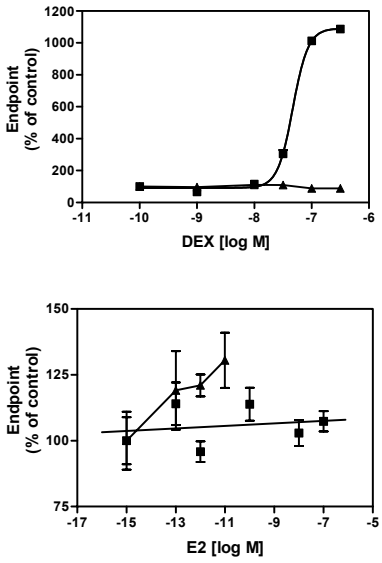


Figure 2 Upper: Primary human mammary fibroblasts cells were co-cultured with MCF-7 cells. Cells were exposed to increasing concentrations of dexamethasone (DEX). ■ represents aromatase activity (as percentage of control) in primary human mammary fibroblasts co-cultured with MCF-7 cells. ▲ represents MCF-7 cell proliferation (as percentage of control).

Lower: Primary human mammary fibroblasts cells were co-cultured with MCF-7 cells. Cells were exposed to increasing concentrations of estradiol (E2). ■ represents aromatase activity (as percentage of control) in primary human mammary fibroblasts co-cultured with MCF-7 cells. ▲ represents MCF-7 cell proliferation (as percentage of control). Each data point represents an average of three measurements. Error bars are SEM.

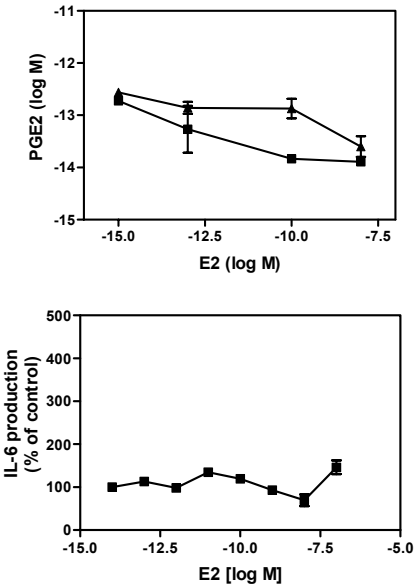


Figure 3 Upper: Concentration of prostaglandin E2 (PGE2) in co-culture medium on day 5 (■) or day 8 (▲).Co-cultures of primary human mammary fibroblasts and MCF-7 cells were exposed to increasing concentrations of estradiol (E2).Each data point represents an average of three measurements. Error bars are SEM

Lower: Concentration of interleukin-6 (as percentage of control) in co-culture medium on day 8. Co-cultures of primary human mammary fibroblasts and MCF-7 cells were exposed to increasing concentrations of estradiol (E2). Each data point represents an average of 3 measurements. Error bars are SEM.

ONE COMPARTMENT CO-CULTURE

Optimization

Pilot experiments showed that the optimal pS2 and aromatase amplification responses were obtained with an incubation time of 72h (data not shown). Furthermore, consistent with a previous study,[6] our MCF-7 cells did not express aromatase; in turn primary human mammary fibroblasts did not express pS2 (data not shown). Also, DEX did not elicit any detectable levels of aromatase gene transcription in MCF-7 cells, nor did estrogens result in detectable pS2 gene transcription in the fibroblasts (data not shown). Exposure of MCF-7 cells to 20 nM of testosterone did not result in a significant increase of pS2 gene transcription (data not shown). Previous studies showed that aromatase activity in primary fibroblasts ranged from 0.005 to 0.09 pmol/h per mg protein in different patients. Also, aromatase gene transcription levels in the fibroblasts differed greatly between batches and varied around the detection limit. As a result we could not consistently use aromatase mRNA transcripts as a parameter in our co-culture experiments, unless aromatase gene transcription was induced by DEX.

Exposure to estrogenic compounds

An exposure of 72 h of the co-culture to E2 (10-100 pM) resulted in a concentration-dependent increase in pS2 gene transcription. The increase was 8-fold compared to solvent control at the highest E2 concentration. Furthermore, in culture medium containing 20 nM of testosterone, E2 increased pS2 gene transcription even further with a maximum increase of 12-fold compared to solvent control without testosterone (figure 4). Aromatase gene transcription was not detectable. A 72 h exposure of the co-culture to the synthetic estrogen DES (30-100 pM) resulted in a concentration-dependent increase in pS2 gene transcription with a maximum of 2.9-fold. When the experiment was performed in culture medium containing 20 nM testosterone, pS2 gene transcription levels were consistently higher than in experiments without testosterone (figure 5). Relative values for pS2 gene transcription levels were significantly higher for cells treated with DES and

testosterone, than for cells treated with DES in the absence of testosterone. Aromatase gene transcription was not detectable.

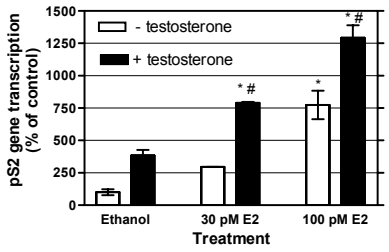
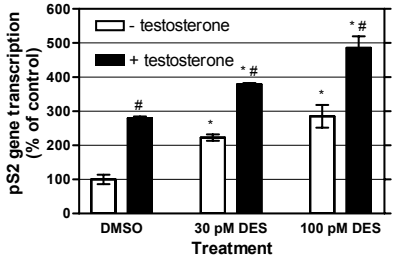


Figure 4: pS2 gene transcription as % of control (ethanol without testosterone) in co-culture of primary human mammary fibroblasts and MCF-7 cells after 72h exposure to 0, 30 or 100 pM E2, with (black bars) or without (white bars) 20 nM testosterone. (*) Significantly different from control (ethanol without testosterone for white bars, ethanol with testosterone for black bars, P<0.01). (#) Significantly different from similar E2-treatment without testosterone (P<0.01). Each bar represents average of triplicate measurements. Error bars represent SEM. Graph shows one representative experiment out of three experiments in tissues from three different samples.

Figure 5: pS2 gene transcription as % of control (DMSO without testosterone) in co-culture of primary human mammary fibroblasts and MCF-7 cells after 72h exposure to 0, 30 or 100 pM DES, with (black bars) or without (white bars) 20 nM testosterone. (*) Significantly different from control (DMSO without testosterone for white bars, DMSO with testosterone for black bars, P<0.05). (#) Significantly different from similar DES-treatment without testosterone (P<0.01). Each bar represents average of triplicate measurements. Error bars represent SEM. Graph shows one representative experiment out of three experiments.



A 72 h exposure of the co-culture to the synthetic estrogen BPA (300 nM–1 µM), also resulted in concentration-dependent increase in pS2 gene transcription. The maximum increase was approximately 2.5-fold in the absence of testosterone. In the presence of 20 nM testosterone and 300 nM or 1 µM BPA resulted in a 1.5- or 2.5-fold induction in pS2 gene transcription compared to solvent control, respectively.

As a control experiment, the co-culture was exposed to 30 µM of the aromatase inhibitor fadrozole (FAD) in addition to BPA and testosterone, resulting in pS2 gene transcription levels similar to those obtained after exposure to BPA only. Again aromatase gene transcription was not detectable (figure 6).

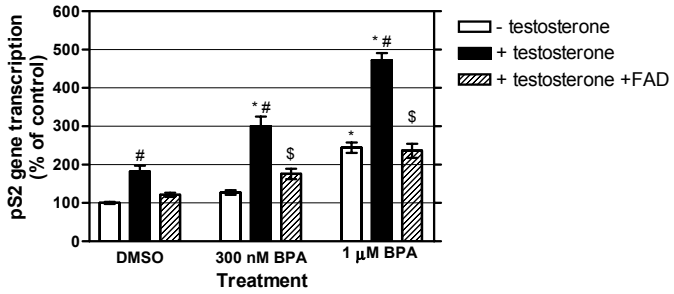


Figure 6: pS2 gene transcription as % of control (DMSO without testosterone) in co-culture of primary human mammary fibroblasts and MCF-7 cells after 72h exposure to 0, 0.3 or 1 µM bisphenol A (BPA, with (black bars) or without (white bars) 20 nM testosterone. Striped bars represent treatment with BPA, 20 nM testosterone and 30 µM fadrozole. testosterone and 30 µM fadrozole. (*) Significantly different from control (DMSO without testosterone for white bar, DMSO with testosterone for black bars, $P<0.001$). (#) Significantly different from similar BPA-treatment without testosterone ($P<0.05$). Each bar represents average of triplicate measurements. ($) Significantly different from similar BPA treatment with 20 nM testosterone ($P< 0.001$). Error bars represent SEM. Graph shows one representative experiment out of four experiments.

Exposure to aromatase inducer dexamethasone

A 72h exposure of the co-culture to DEX (30-100 nM) resulted in a concentration-dependent increase in pS2 gene transcription with maximum values of 2-fold and 5-fold for experiments without testosterone and with 20 nM testosterone,

respectively (figure 7A). Aromatase gene transcription was elevated approximately 3.5-fold for DEX-treatments with and without 20 nM testosterone (figure 7B).

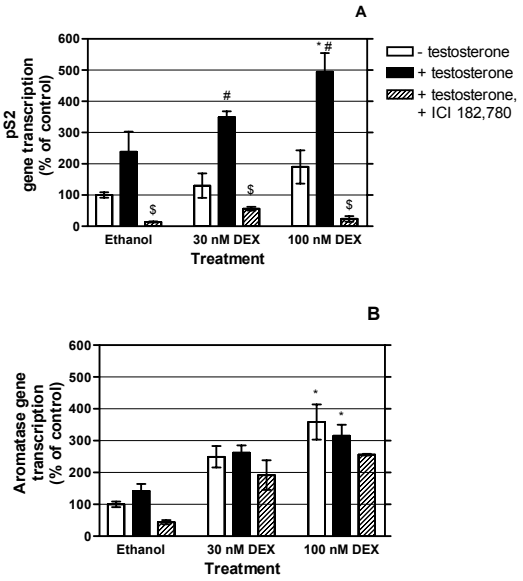


Figure 7: A: pS2 gene transcription as % of control (ethanol without testosterone) in co-culture of primary human mammary fibroblasts and MCF-7 cells after 72h exposure to 0, 30 or 100 nM DEX, with (black bars) or without (white bars) 20 nM testosterone. Striped bars represent treatment with DEX, 20 nM testosterone and 10 nM ICI 182,780. (*) Significantly different from ethanol with testosterone (P<0.01). (#) Significantly different from similar DEX-treatment without testosterone (P<0.01). ($) Significantly different from similar DEX-treatment with 20 nM testosterone (P< 0.05). Each bar represents average of triplicate measurements. Error bars represent SEM. **B:** Aromatase gene transcription as % of control (ethanol without testosterone) in co-culture of primary human mammary fibroblasts and MCF-7 cells after 72h exposure to 0, 30 or 100 nM DEX, with (black bars) or without (white bars) 20 nM testosterone. Striped bars represent treatment with DEX, 20 nM testosterone and 10 nM ICI 182,780. (*) Significantly different from control (ethanol without testosterone for white bar, ethanol with testosterone for black bar (P<0.05). Each bar represents average of triplicate measurements. Error bars represent SEM.

As a control, cells were exposed to 10 nM of the synthetic anti-estrogen ICI 182,780 in addition to DEX and testosterone. When ICI 182,780 was added at a concentration of 10 nM, pS2 gene transcription was almost completely blocked. Aromatase gene transcription in this case appeared lower than in samples with the same DEX- and testosterone treatment without ICI 182,780 (figure 7B), but this decrease was not statistically significant.

When cells in the co-culture were exposed to FAD in addition to DEX and testosterone, pS2 gene transcription was almost completely blocked, except for the treatments without DEX (figure 8). Previous experiments have shown that 30 µM FAD blocked aromatase enzyme activity in both H295R and R2C cells.[8]

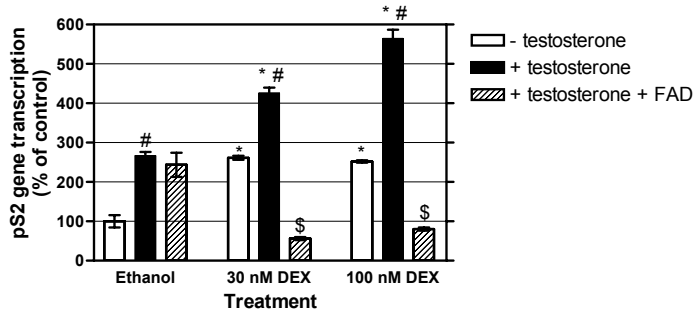


Figure 8: pS2 gene transcription as % of control (ethanol without testosterone) in co-culture of primary human mammary fibroblasts and MCF-7 cells after 72h exposure to 0, 30 or 100 nM dexamethasone (DEX), with (black bars) or without (white bars) 20 nM testosterone. Striped bars represent treatment with DEX, 20 nM testosterone and 30 µM fadrozole). (*) Significantly different from control (ethanol without testosterone for white bars, ethanol with testosterone for black bars, P <0.001). (#) Significantly different from similar DEX-treatment without testosterone (P <0.001). ($) Significantly different from similar DEX-treatment with 20 nM white testosterone (P < 0)

Discussion

Isolation of human mammary fibroblasts

The method for fibroblast isolation was derived from a protocol developed by Van de Ven and co-workers.[26] Some major adjustments were made in order to optimize fibroblast isolation. First, during transportation of the tissue to our laboratory, tissue was kept in ice-cold sterile PBS. Second, 150 U/ml collagenase I was dissolved in Krebs' Ringer buffer instead of PBS. Collagenase requires calcium for its activation and Krebs' Ringer buffer does contain calcium in contrast to PBS. Tissue digestion lasted for 2.5h instead of 30-45 min. These modifications were believed to greatly enhance cell yields allowing us to use the cells at passage number 2 or 3 after 3 weeks instead of at passage number 4-5 after 9 weeks. Cells at low passage numbers showed significantly higher levels of aromatase activity than cells at higher passage numbers (personal communication Dr. J. van de Ven).

TWO COMPARTMENT CO-CULTURE

Co-cultures of primary human mammary fibroblasts and MCF-7 cells were exposed to increasing concentrations (1-300 nM) of the synthetic glucocorticoid dexamethasone (DEX) to evaluate effects on aromatase activity in the fibroblasts and subsequent MCF-7 cell proliferation. We expected that aromatase activity would increase concentration-dependently and that MCF-7 cells would proliferate as a result of increased estrogen production by the fibroblasts. Aromatase activity increased concentration-dependently, with a maximum effect of 11-fold induction at the highest tested concentration of 300 nM DEX. However, no effects on MCF-7 cell proliferation were observed. When co-cultures were exposed to increasing concentrations of E2 (0.1 pM – 100 nM), we expected MCF-7 cells to proliferate concentration-dependently and that aromatase activity in fibroblasts would increase as a result of increased secretion of IL-6, IL-6sR or PGE2 by the MCF-7 cells. MCF-7 cell proliferation increased concentration-dependently up to a concentration of 10 pM. At higher concentrations cell counts dropped drastically, probably due to a E2-mediated cytotoxicity. No effects on aromatase activity were observed in the

fibroblasts. These results suggest that in this two-compartment system, these compounds only had a direct effect on their target cells. Paracrine interactions between the two cell types could not be established. A possible reason for the absence of these interactins could be found in the large volume of culture medium that was needed to supply both the fibroblasts on the bottom of the well and the MCF-7 cells in the inserts with nutrients.

PGE2 and IL-6

Driven by the results obtained with the positive controls DEX (for aromatase activity in fibroblasts) and E2 (for MCF-7 cell proliferation), we attempted to quantify concentrations of PGE2 and IL-6 that were secreted by the MCF-7 cells into the medium. We expected that upon E2-stimulation, both IL-6 and PGE2 secretion would increase concentration-dependently but this effect was not observed. In contrast to our expectations, PGE2 levels even decreased with increasing E2-concentrations. However, concentrations were higher at day 6 than at day 3 (figure 7). PGE2 concentrations varied around 0.1 pM, which is much lower than the 1 µM that elicited induction of aromatase activity in fibroblasts in the study described in chapter 4 of this thesis. Also IL-6 levels did not increase significantly after E2 exposure with concentrations varying around 2000 U/ml, which corresponds with a concentration of 40 ng/ml. In another study, it was found that exposure of primary human mammary fibroblasts to 50 ng/ml IL-6 resulted in 27% increase of aromatase activity,[27,28] indicating that the concentration in our co-culture should be high enough to elicit a slight increase in aromatase activity. As reported previously,[21] the great variability of basal and inducible aromatase activity between persons can account for differences in responses obtained in tissues from different patients.

We concluded that paracrine interactions could not be established in these two compartment co-cultures of primary human mammary fibroblasts and MCF-7 cells, under the described experimental conditions. We consider the large volume of culture medium that was required when using these 6-well plates with inserts, as a very likely reason for the observed absence of paracrine interactions between the fibroblasts and the MCF-7 cells. Secreted factors that should be responsible for

these paracrine interactions were probably diluted to such an extent that their concentrations were too low to elicit an effect. Furthermore, intimate cell-cell contact might be another prerequisite to establish measurable paracrine effects. Therefore, we changed the experimental design in which both cell types were cultured in one compartment. This implicated that other endpoints than aromatase activity and cell proliferation had to be chosen, as it was impossible to separate both cell types after incubation. Consequently, we decided to use pS2 gene transcription as a marker for estrogenicity in the MCF-7 cells and aromatase gene transcription to assess effects on aromatase in the fibroblasts. The pS2 gene was originally identified as an estrogen-inducible transcript in MCF-7 cells [29] and pS2-gene expression is an adequate way to assess estrogenicity in humans.[30]

ONE COMPARTMENT CO-CULTURE

Regulation of aromatase gene transcription

Aromatase gene transcription is regulated in a tissue-specific manner, using alternate promoter regions. In healthy breast adipose tissue, the predominant aromatase promoter region I.4 is regulated by glucocorticoids and class I cytokines via the Jak1/STAT3 signaling pathway.[31] Previously, we have shown that exposure of fibroblasts to DEX increases promoter I.4 specific aromatase gene transcription and subsequent enzyme acitivity.

In breast tumors, it has been found that aromatase promoter regions I.3 and pII become more active. These promoter regions are stimulated by cyclic adenosine monophosphate (cAMP) through the protein kinase A (PKA) and by phorbol esters through the protein kinase C (PKC) phosphorylation pathways. Exposure of human adrenocortical carcinoma H295R cells to PGE2 resulted in elevated levels of promoter I.3 and pII-specific aromatase gene transcription.[8] Prostaglandin E2 (PGE2) is a major product secreted by breast tumor epithelial cells [32] and thought to be the most important stimulator of the I.3/pII pathway [33] by stimulating both PKA and PKC phosphorylation pathways. PGE2 interacts with two receptor subtypes in fibroblasts, EP_1 and EP_2. Upon binding of PGE2 to EP_1, the associated G protein

stimulates phospholipase C, resulting in formation of second messengers and activation of PKC. Subsequently, a cascade of serine/threonine phosphorylation events is initiated.[32] Upon binding of PGE2 to EP_2, the associated G protein stimulates adenylyl cyclase activity, resulting in elevated production of cAMP. Subsequently, the cAMP responsive element (CRE) in promoter regions I.3 and pII binds CRE binding protein (CREB) which is phosphorylated via PKA-mediated pathways.[34] As a consequence, the stimulation elicited by PGE2 is equivalent to the combined response of forskolin (enhancer of cAMP production, which activates PKA) and phorbol esters (which are activators of PKC).[33]

Furthermore, PGE2 appears to stimulate IL-6 production in fibroblasts,[12] leading to increased expression of pII/I.3-derived CYP19 transcripts as well as that of I.4. IL-6 was shown only to be present in small quantities (<10 ng/ml) in breast cyst fluids and at these low levels, aromatase activity was not elevated in cultured breast fibroblasts. However, if IL-6sR was added to fibroblasts, aromatase activity was enhanced markedly.[35] ER-positive MCF-7 and T47D cells have been shown to secrete IL-6sR after E2 or DEX stimulation in contrast to ER-negative MDA-MB-231 cells.[18,36] Based on these studies we suggest that an increased IL-6sR secretion by MCF-7 cells is a possible mechanism explaining the elevated concentration-dependent increase of pS2 gene transcription in our co-culture experiments after exposure to (xeno)estrogens in the presence of testosterone.

Exposure of the co-culture to dexamethasone

Addition of DEX to fibroblasts increased aromatase activity significantly. Stimulation of aromatase activity by DEX was almost two-fold higher when fibroblasts were co-cultured with MCF-7 cells.[37] The increased stimulation of aromatase activity in these co-cultures is consistent with the possibility of a factor being secreted by MCF-7 cells which has a paracrine stimulatory effect on aromatase activity in fibroblasts.[37]

In this study, co-cultures exposed to increasing concentrations of DEX had elevated aromatase activity and gene transcription levels. This confirms observations by Quinn and coworkers, who demonstrated that the culture medium removed from

fibroblasts that were exposed to DEX and testosterone, elicited a pS2 response in MCF-7 cells.[38] Additionally, DEX might have increased IL-6sR secretion by the MCF-7 cells,[18] stimulating gene transcription via promoter region I.4 even further.

Aromatase gene transcription was not further elevated by addition of testosterone, which was expected since testosterone is a substrate for the aromatase enzyme and not an inducer of aromatase gene transcription, nor did ICI 182,780 alter aromatase gene transcription. In the presence of testosterone, however, pS2 gene transcription was increased even further. These results indicate that testosterone served as substrate for aromatase in the fibroblasts, resulting in elevated estradiol production which subsequently further increased pS2 gene transcription. ICI 182,780 completely blocked pS2 gene transcription confirming ER-mediated regulation of pS2 expression. Anti-estrogens have been shown to inhibit E2-stimulated IL-6sR secretion by MCF-7 cells,[18] resulting in less pronounced induction of aromatase gene expression (figure 9).

Exposure to estrogenic compounds

MCF-7 cells are stimulated by E2, DES and BPA, resulting in increased pS2 gene transcription and cell proliferation.[30,39,40]

Although 300 nM of the environmental contaminant BPA did not increase pS2 gene transcription in the absence of testosterone, addition of testosterone to the medium, which is more likely to reflect the *in vivo* situation, increased pS2 gene transcription by about 1.5 fold at the same BPA concentration. Higher levels of pS2 gene transcription after addition of testosterone to the medium are to be contributed to aromatase enzyme activity in the fibroblasts, since addition of FAD reduces pS2 transcription to the same level as without testosterone, but not to the lower levels that were observed after exposure to the ER-antagonist ICI 182,780.

Compounds with estrogenic properties are able to induce cell proliferation of the MCF-7 cells directly by activation of the ER. Furthermore, they can also induce IL-6sR secretion by MCF-7 cells, which leads to elevated aromatase gene transcription and subsequent synthesis of E2. E2-dependent IL-6sR secretion is also

an ER-mediated event, which is completely blocked by the anti-estrogen 4-hydroxytamoxifen.[18]

A greater number of MCF-7 cells secrete higher levels of PGE2, IL-6 and IL-6sR through which aromatase gene transcription is elevated. Various compounds can interfere with the feedback loop (figure 9). DEX induces aromatase gene expression directly via promoter region I.4 [10] but DEX also elevates IL-6sR secretion by MCF-7 cells [18,36] resulting in increased stimulatory signals for aromatase promoter region I.4 in the adjacent fibroblasts.

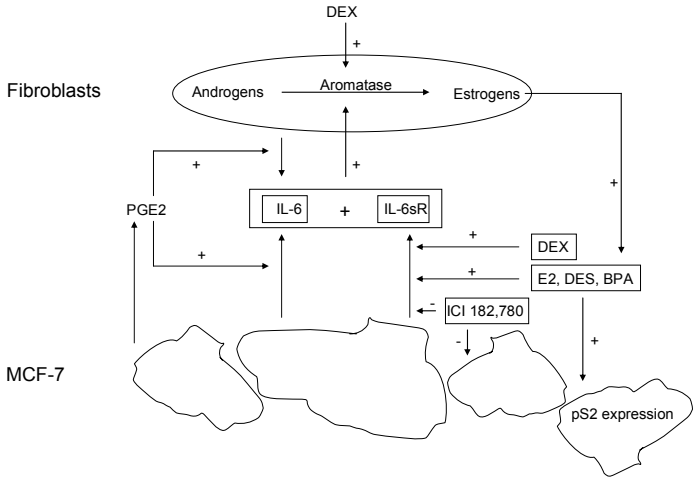


Figure 9: Schematic representation of proposed positive feedback loop in the presence of an estrogen-dependent breast tumor. MCF-7 (epithelial tumor) cells are stimulated by estrogens, resulting in cell proliferation. MCF-7 cells secrete higher levels of PGE2, IL-6 and IL-6sR which in turn elevate aromatase gene transcription in surrounding (primary) fibroblasts. Compounds with estrogenic properties (estradiol (E2), diethylstilbestrol (DES), bisphenol A (BPA)) are able to induce cell proliferation of the MCF-7 cells directly by binding to the ER. Furthermore, they can induce IL-6sR secretion by MCF-7 cells as well, which leads to elevated aromatase gene transcription and subsequent production of E2. Anti-estrogens, such as ICI 182,780 inhibit all actions of estrogens by blocking the ER. DEX not only induces aromatase gene expression directly via promoter region I.4, but also elevates IL-6sR secretion by MCF-7 cells resulting in increased stimulatory signals for aromatase promoter region I.4 in the fibroblasts.

Positive feedback loop

The consistently higher levels of pS2 gene transcription after exposure of the co-culture to estrogenic compounds in the presence of 20 nM testosterone compared to the same estrogen-treatment without addition of testosterone, suggests the presence of a feedback loop, which is established by intercellular communication between epithelial MCF-7 cells and fibroblasts.

To our knowledge, this is the first study in which the positive feedback loop between human breast cancer cells and primary human mammary fibroblasts has been established. Our observation that estrogenic effects caused by xenoestrogens, such as BPA, are potentiated in a co-culture of both MCF-7 cells and fibroblasts require reassessment of the endocrine disrupting potential of these xenobiotics. Furthermore, compounds that can stimulate aromatase expression and aromatase enzyme activity have to be taken into account when looking for potential endocrine disruptors and possible promoters of already existing breast tumors. These compounds could elicit indirect estrogenic effects and tumor promotion. A co-culture test system of tumor cells and fibroblasts will provide a more realistic and more sensitive test system for direct and indirect estrogenic effects of chemicals.

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Estrogenic effects of frequently used UV-filters in a co-culture of primary human mammary fibroblasts and MCF-7 cells

6

Marjoke Heneweer, Sandra M. Nijmeijer, Milou M.L. Dingemans, Martin van den Berg, and J. Thomas Sanderson

Institute for Risk Assessment Sciences (IRAS), Utrecht University, PO Box 80176, 3508 TD Utrecht, The Netherlands

Abstract

Approximately 60% of all breast tumors are estrogen-responsive and chemicals that show estrogenic or anti-estrogenic properties are able to interact with breast tumor growth. In a breast tumor, adipose stromal cells (fibroblasts) surrounding the epithelial tumor contain the enzyme aromatase, which converts androgens into estrogens. Exposure to aromatase inducers can therefore lead to increased estrogen levels and possibly accelerate breast tumor growth. Subsequently, breast tumor cells synthesize and secrete elevated levels of factors such as prostaglandin E2 (PGE2), interleukin-6 (IL-6) and IL-6 soluble receptor (IL-6sR), which in turn have the ability to stimulate aromatase gene transcription in surrounding fibroblasts, establishing a positive feedback loop. In this study, a technique that allows for co-culturing MCF-7 epithelial breast tumor cells and healthy primary human mammary fibroblasts was used. To establish this possible positive feedback loop, the co-culture was exposed to benzophenones from which an estrogen receptor-mediated mechanism of action was expected. Estrogen-regulated pS2 gene transcription in the MCF-7 cells was chosen as endpoint. RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR) were performed. The UV-filter BP-3 and its metabolites BP-1 and BP-4 (10 µM), all stimulated pS2-gene transcription in the absence of androgen substrate for aromatase, indicating that the observed effect on pS2-gene transcription was directly ER-mediated. Estrogenic potencies were at least 5 orders of magnitude lower than that observed for 17β-estradiol (E2). Only cells exposed to different concentrations of BP-1 showed consistently higher pS2-gene transcription levels when grown in medium supplemented with testosterone as an aromatase substrate. When culture medium was supplemented with 20 nM testosterone and 30 µM of the non-steroidal aromatase inhibitor FAD, all compounds were able to induce pS2-gene transcription. When culture medium was supplemented with 20 nM testosterone, as well as 10 nM of the synthetic anti-estrogen ICI 182,780, pS2-gene transcription was completely blocked despite exposure to the UV-filters or E2. Exposure of MCF-7 cells to 50 µM of BP-1 and BP-3 did not induce DNA-damage in MCF-7 cells after 4h exposure. In conclusion, the tested compounds elicited an estrogenic effect in the MCF-7 cells, but BP-3 and BP-4 were not potent enough to induce the positive feedback loop between the fibroblasts and MCF-cells.

Introduction

In the protection against harmful ultraviolet (UV)-radiation, many people use commercial sunscreens when exposed to sunlight. Many of these sunscreens contain organic UV-filters that absorb UV-A (400-315 nm) and UV-B (315-280 nm) radiation.[1] The use of sunscreens has increased significantly over the past years due to concerns about skin damage by UV-light. Toxicological classification of UV-filters varies in different parts of the world. In the US, they are classified as over-the-counter drugs and in the European Union as cosmetic ingredients. Japan classifies these compounds either as cosmetics or quasi-drugs.[1]

Not only sunscreens, but also creams, lipsticks, lotions, and shampoos contain detectable amounts of these compounds. They have been added for stability and durability. Many UV-filters are lipophilic and can accumulate in humans and the environment. In perch (*Perca flavescens*) and roach (*Rutilus rutilus*), six different UV screens have been detected at concentrations of 2 mg/kg lipid and 0.5 mg/kg lipid, respectively.[1] Both fish species were contaminated with UV-filters at similar levels as PCBs and DDT and therefore UV-filters could be considered to be relevant environmental contaminants in the aquatic environment. Human exposure can occur through dermal absorption, [2-4] as well as through the food chain. UV-filters have been detected in human urine 4 hr after application of commercial sunscreen products to the skin. Moreover, they are readily absorbed from the GI tract [5] and benzophenone-3 (BP-3) and octyl methoxy cinnamate (OMC) have been detected in human milk.[6]

The fact that many of these compounds are lipophilic and are able to bioaccumulate, make these compounds interesting subjects of research in long-term studies. Schlumpf and co-workers reported estrogenic effects of these compounds in different *in vitro* and *in vivo* tests.[1] Exposure to UV-filters caused MCF-7 cell proliferation and peroral administration and dermal application of the UV filters caused an increase of uterine weight in immature rats. Experiments in our lab showed that several UV-filters elicited increased pS2-gene transcription in MCF-7 cells. Although UV-filters were 5 orders of magnitude less potent than E2, the

maximum effect that was elicited was similar, indicating they acted as full agonists for the ER except for BP-3.[7]

Since approximately 60% of all breast tumors are estrogen-responsive,[8,9] chemicals that show estrogenic properties are able to interfere with breast tumor growth. Epithelial breast tumor cells express estrogen receptors (ERs) to which endogenous and/or exogenous estrogens can bind. These compounds may act either as ER-agonists or antagonists, causing subsequent increased or decreased estrogenic effects, respectively in the cells. Estrogens in a breast tumor are mainly formed in the surrounding stromal cells (fibroblasts). These cells contain the enzyme aromatase, which is responsible for converting androgens into estrogens. In previous experiments we have co-cultured MCF-7 cells with primary human mammary fibroblasts (see chapter 5).[10] In this study, paracrine interactions were established between the two cell types, resulting in enhanced estrogenic effects of known estrogens when culture medium was supplemented with an androgen substrate for aromatase. In the case of breast cancer, epithelial tumor cells secrete among others class I cytokines [11,12] and prostaglandin E2 (PGE2) [9] that diffuse into the fibroblasts surrounding the tumor. Subsequently, these factors are able to induce aromatase activity in the fibroblasts in a paracrine manner. Induced aromatase activity in the fibroblasts leads to locally increased estrogen synthesis. Subsequently, these estrogens then diffuse into the epithelial tumor cells and further stimulate proliferation of the tumor cells. In this way a positive feedback loop between epithelial breast tumor cells and fibroblasts is established.

In the present study, we exposed co-cultures of MCF-7 cells and primary human mammary fibroblasts to different concentrations of the UV-filter 2-hydroxy-4-methoxybenzophenone (BP-3), or its metabolites 2-4-dihydroxybenzophenone (BP-1) and 4-hydroxybenzophenone (BP-4) (figure 1). The co-cultures were grown in culture medium with or without testosterone as the substrate for aromatase. We hypothesized that pS2-gene transcription could increase after exposure to increasing concentrations of UV-filters. Furthermore, we expected that supplementation of the culture medium with testosterone would increase pS2-gene transcription to higher levels compared to the unsupplemented control situation. In addition to estrogenic

effects of the UV-filters, we also wanted to study the possible tumor-initiating effects of these compounds. As there were conflicting reports [13,14] on the carcinogenic effects of benzophenones, we also performed a alkaline single-cell gel electrophoresis (comet) assay in order to study possible genotoxic effects of these compounds.

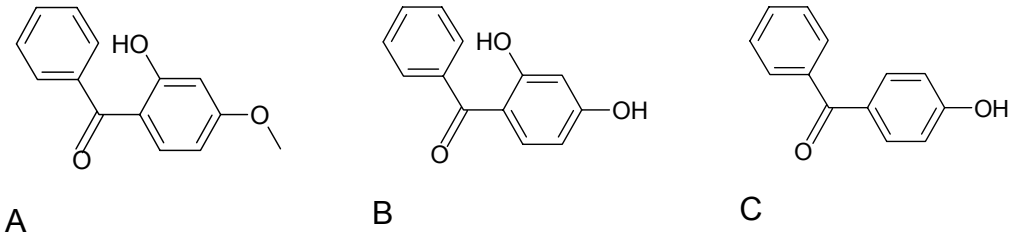

Figure 1 A: 2-hydroxy-4-methoxybenzophenone (BP-3). **B:** 2,4-dihydroxybenzophenone (BP-1). **C:** 4-hydroxybenzophenone (BP-4).

Methods

MCF-7 cell culture

The MCF-7 malignant human mammary epithelial cancer cell line was obtained from the American Type Culture Collection (ATCC # HTB-22) and cultured in phenol red-free RPMI 1640 medium containing L-glutamine (GibcoBRL 11835-030). The culture medium was supplemented with 10% heat-inactivated fetal calf serum (FCS), 100U/l penicilline/100 µg/l streptomycin (GibcoBRL 15140-114) and 1 µg/ml insulin (Sigma, St. Louis, MO, USA). Prior to assays, cells were placed on medium containing stripped FCS.

Isolation and co-culture of fibroblasts and MCF-7 cells

Breast tissue was collected from the St. Antonius Hospital in Nieuwegein from women undergoing reduction mammoplasty, after informed consent (TME/Z-02-09, Medical Ethical Committee, St. Antonius Hospital, Nieuwegein, The Netherlands). Tissue was pathologically classified as healthy. Human mammary fibroblasts were isolated and used in co-culture with MCF-7 cells according to previously described methods.[15]

RNA extraction and RT-PCR

After exposure to the test compounds for 24h, RNA was isolated from co-cultured cells using the RNA Instapure System (Eurogentec, Liège, Belgium) according to the enclosed instruction. Purity of RNA was checked by measuring 260/280 nm wavelength absorption ratio. RNA was stored in –70 °C in aliquots of 50 ng/µl.

Primers coding for the estrogen-responsive pS2 gene were used after Lee.[16] RT-PCR was performed using the Access RT-PCR System (Promega, Madison, WI, USA) according to previously described methods[7,10] Amplification products were detected using 2% agarose gel electrophoresis and ethidium bromide staining. Intensity of the stains was quantified using a FluorImager (Molecular Dynamics, USA).

Alkaline single-cell gel electrophoresis (comet) assay

MCF-7 cells ($1.75x10^5$ cells/ml) were transferred to 12-wells plates (2 ml/well) in steroid-free medium, 24h prior to assay. The comet assay was performed according to Singh et al.,[17] with minor modifications. To prepare the slides, they were placed in methanol (MeOH) overnight. Slides were dipped in normal melting agarose (NMA, 1.5 g/100 ml PBS), which was heated in a microwave. After 12h on ice, slides were ready to be used. Frosted microscope slides were covered on one side with 1.5% NMA. After 4 hours of incubation with test compounds, low melting agarose (0.5% LMA) was added to the single cell suspension at 37 ˚C and spread onto the slide. A cover slip was placed on top of the suspension and the slides were placed on ice. For lysis of the cells and nucleus membranes, cover slips were removed and slides were placed in freshly prepared cold lysis solution (2.5M NaCl, 0.1M Na_2EDTA, 10 mM Tris, supplemented with 1% Triton-X-100, pH 10) at 4 °C for 1 h. To avoid any additional DNA damage, the following steps were performed in darkness. For denaturation, slides were placed in a horizontal slide holder in cold electrophoresis solution (0.3M NaOH, 0.001M EDTA, approx. 10 ˚C) for 30 min. Electrophoresis was performed for 20 min at ~25V and 290-310 mA. For neutralization, the slides were washed three times with a solution of 0.4M Tris/HCl, pH 7.5. For dehydratation, slides were put in 96% ethanol for 10 min and stored until analysis. For analysis, slides were stained with ethidium bromide (20 µg/l). Digital pictures were made under a fluorescence microscope (20x objective, excitation filter 500 nm) and analyzed using comet assay software program CASP[18] Tail moment (Comet tail length X % tail DNA) was calculated for each treatment. As a positive control a 1 min exposure of the cells to UV light was used, which does not necessarily result in maximal DNA damage. Results obtained after BP-1 or BP-3 exposure can therefore not be compared with the positive control in a quantitative manner.

Data analysis

In co-culture experiments, each concentration was tested in triplicate. Human tissue samples were not pooled, therefore each sample served as its own control. For graphs and statistical analyses, Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA) was used. All error bars represent standard error of the mean (SEM). To identify statistically significant differences among means, a one-way ANOVA, followed by Tukey-Kramer's posteriori-test was performed for co-culture experiments and a students t-test for the comet assay.

Results

Estrogenicity

When the culture medium was not supplemented with testosterone, BP-1, BP-3 and BP-4 all elevated pS2-gene transcription in the co-culture at a concentration of 10 µM. At this concentration BP-1, BP-3 and BP-4 induced pS2-gene transcription 6-fold, 2-fold and 4-fold, respectively. E2 elicited a 6-fold induction of pS2-gene transcription at a concentration of 30 pM (figure 2).

When the culture medium was supplemented with 20 nM testosterone as a substrate for aromatase, again all compounds enhanced pS2-gene transcription at a concentration of 10 µM. BP-1, BP-3 and BP-4 induced pS2-gene transcription 7.5-fold, 2-fold and 2.5-fold, respectively. E2 induced pS2-gene transcription 4-fold at the highest tested concentration of 100 pM (figure 3).

When the culture medium was supplemented with 20 nM testosterone and 30 µM of the non-steroidal aromatase inhibitor FAD, all compounds were again able to induce pS2-gene transcription. At a concentration of 10 µM, BP-1, BP-3 and BP-4 induced pS2-gene transcription 2-fold, 1.5-fold and 4-fold, respectively. 30 pM E2 caused a 7-fold increase in pS2-gene transcription (figure 4).

When culture medium was supplemented with 20 nM testosterone, as well as 10 nM of the synthetic anti-estrogen ICI 182,780, induction of pS2-gene transcription by UV-filters or E2 was completely blocked (figure 5).

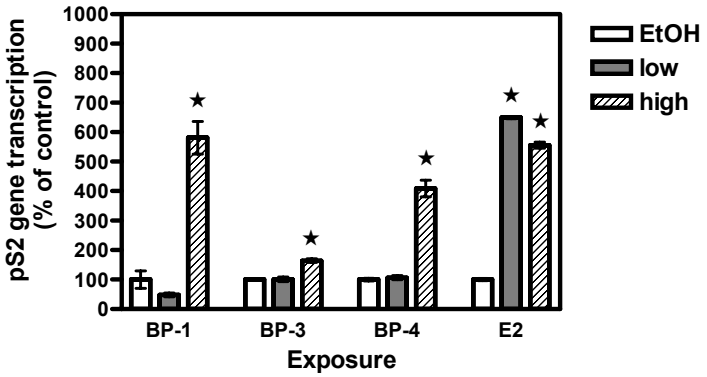

Figure 2: pS2-gene transcription in MCF-7 cells as % of control (EtOH exposed cells). Cells were exposed to low (1 µM for UV-filters and 30 pM for E2, dotted bars) or high (10 µM for UV-filters and 100 pM for E2, striped bars) concentrations of benzophenone-1 (BP-1), benzophenone-3 (BP-3), benzophenone-4 (BP-4) or E2. ★Significantly different from control (P < 0.001). Bars represent averages of triplicate measurements. Error bars represent SEM.

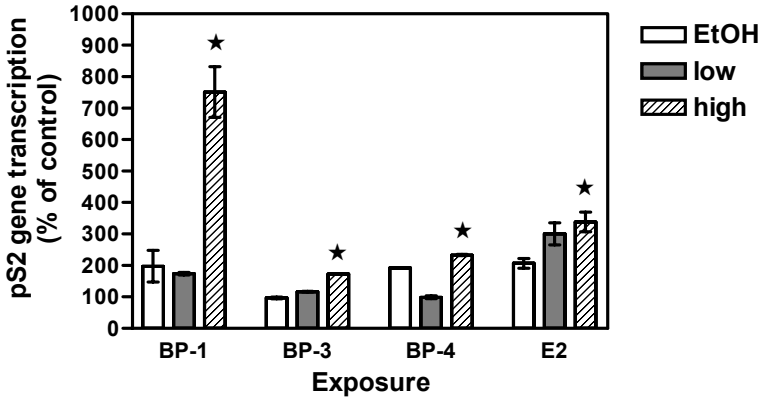

Figure 3: pS2-gene transcription in MCF-7 cells as % of control (EtOH exposed cells, unsupplemented medium, see figure 1). Cells were exposed to low (1 µM for UV-filters and 30 pM for E2, dotted bars) or high (10 µM for UV-filters and 100 pM for E2, striped bars) concentrations of benzophenone-1 (BP-1), benzophenone-3 (BP-3), benzophenone-4 (BP-4) or E2. Medium was supplemented with 20 nM testosterone. ★ Significantly different from control (P < 0.001, P < 0.05 for E2). Bars represent averages of triplicate measurements. Error bars represent SEM.

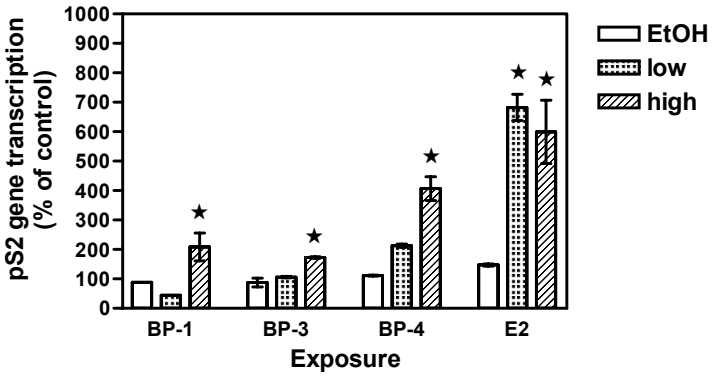

Figure 4: pS2-gene transcription in MCF-7 cells as % of control (EtOH exposed cells, unsupplemented medium, see figure 1). Cells were exposed to low (1 µM for UV-filters and 30 pM for E2, dotted bars) or high (10 µM for UV-filters and 100 pM for E2, striped bars) concentrations of benzophenone-1 (BP-1), benzophenone-3 (BP-3), benzophenone-4 (BP-4) or E2. Medium was supplemented with 20 nM testosterone and 30 µM fadrozole. ★Significantly different from control ($P < 0.001$ for BP-3 and E2; $P < 0.001$ for BP-4 and $P < 0.05$ for BP-1). Bars represent averages of triplicate measurements. Error bars represent SEM.

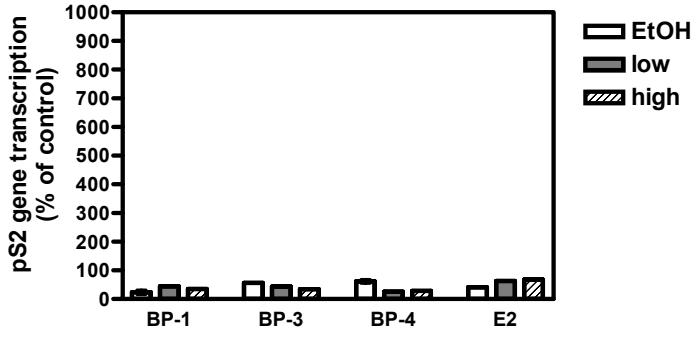

Figure 5: pS2-gene transcription in MCF-7 cells as % of control (EtOH exposed cells, unsupplemented medium, see figure 1). Cells were exposed to low (1 µM for UV-filters and 30 pM for E2, dotted bars) or high (10 µM for UV-filters and 100 pM for E2, striped bars) concentrations of benzophenone-1 (BP-1), benzophenone-3 (BP-3), benzophenone-4 (BP-4) or E2. Medium was supplemented with 20 nM testosterone and 10 nM ICI 182,780. Bars represent averages of triplicate measurements. Error bars represent SEM.

Alkaline single-cell gel electrophoresis (comet) assay

UV-light was used as positive control, which resulted in extensive DNA damage. Exposure of MCF-7 cells to 50 µM of BP-1 and BP-3 did not induce statistically significant DNA-damage in MCF-7 cells after 4h exposure compared to the negative control (*P* >0.05) (figure 6).

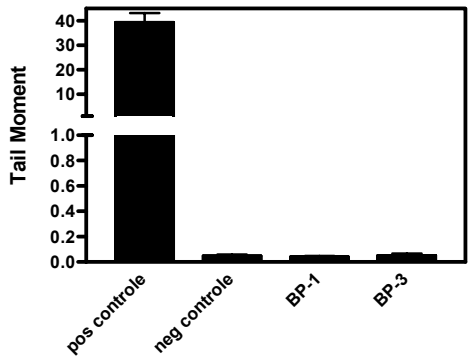

Figure 6: DNA damage in MCF-7 cells after exposure to 50 µM benzophenone-1 or benzophenone-3. Positive control was 1 min UV-light exposure and ethanol served as negative control. Bars represent mean Tail Moment (comet tail length x % of total DNA in comet tail). Error bars represent SEM.

Discussion

Estrogenicity

In our experiments it was shown that all tested compounds stimulated pS2-gene transcription in the co-culture of MCF-7 cells and primary human mammary fibroblasts (figure 2). The observation that this induction was not altered by addition of testosterone as a substrate for aromatase, suggests that the observed effect was a direct effect on estrogen-mediated pS2-gene transcription. From the three UV-filters that were studied, only BP-1 was able to induce pS2-gene transcription as to the same extent as E2. However, E2 elicited a 6-fold induction at a concentration of 30 pM, while BP-1 caused a similar effect at a much higher concentration of 10 μM. This indicates a difference in estrogenic potency of at least 5 orders of magnitude, although full concentration-response curves would need to be produced for more accurate assessment. BP-3 and BP-4 were found to have a much lower estrogenic potency than BP-1 as at 10 μM neither reached a level of pS2-gene transcription even close to the maximum induction level observed for E2. This observation suggests that either even higher concentrations are needed to reach a similar effect or that these compounds are partial agonists for the ER. A previous study in our lab also suggested that BP-3 may be a partial agonist for the ER, causing sub maximal pS2-gene transcription in MCF-7 monocultures.[7] In a yeast estrogen assay, in which yeast cells were transfected with the human estrogen receptor α (ERα) gene, together with expression plasmids containing estrogen responsive elements and the *lac-Z* reporter gene, BP-3 elicited sub maximal estrogenic activity at a concentration of 30 μM.[19] However, BP-3 was reported to be a full ER-agonist in MCF-7 cell proliferation experiments [1] and in *in vitro* reporter gene assays using HEK293 cells with stably transfected ER and luciferase.[20]

When culture medium was supplemented with 20 nM of testosterone, we expected pS2-gene transcription levels to be consistently higher than the levels observed in co-cultures that were grown in unsupplemented medium. This effect has been observed in a previous study where co-cultures were exposed to known estrogenic compounds.[10] Based on these earlier results, it was hypothesized that aromatase in

the fibroblasts would convert testosterone into estradiol and subsequently elicit an additional increase in pS2-gene transcription. Only cells that were exposed to BP-1 showed consistently higher pS2-gene transcription levels when grown in supplemented medium. In our experiments with BP-3 and BP-4 this effect was not observed. Possibly, BP-3 and BP-4 were not potent enough to cause an indirect, paracrine effect in the fibroblasts and induce aromatase activity. However, in this case, a paracrine effect was expected after exposure to the endogenous and potent ER-ligand E2, but this effect was also not observed. Another explanation is that basal aromatase activity was low and poorly inducible in the fibroblasts used for this experiment. Previous studies have shown that aromatase activity and inducibility vary greatly among patients.[15,21] Clearly, further experiments with larger sample sizes and full concentration-response curves should be performed in order to draw more solid conclusions.

When culture medium was supplemented with 20 nM testosterone and 30 µM of the non-steroidal aromatase inhibitor fadrozole, it was expected that fadrozole would abolish the effect of testosterone-addition. pS2-gene transcription levels would be similar to the levels that were observed in the absence of testosterone. For all compounds except BP-1, this was the case. Exposure to BP-1 resulted in lower pS2-gene transcription levels than observed when culturing in the unsupplemented medium. However, an increase in pS2-gene transcription when testosterone was present in the medium was not observed (figure 3), indicating that paracrine activation of aromatase in the fibroblasts has not taken place. The absence of paracrine interactions prevented us from detecting the aromatase-inhibiting effects of fadrozole.

Our experiments clearly show that induction of pS2-gene transcription by UV-filters is ER-mediated (figure 5). The synthetic anti-estrogen ICI 182,780 completely blocked pS2-gene transcription, even to levels lower than basal levels that were observed in cells only exposed to EtOH.

Benzophenones as aromatase inhibitor

Another possible reason for the absence of paracrine interactions in our co-culture of MCF-7 cells and primary human mammary fibroblasts could be a direct effect of the benzophenones on aromatase. Vaz and colleagues [22] reported competitive aromatase inhibition in human placental microsomes after exposure to benzophenones. However, the apparent Ki value of 130 µM is rather high and therefore, it is not likely that aromatase inhibition by BP-1, BP-3 and BP-4 played a significant role in disturbing the paracrine interactions between the two cell types in our co-culture, regarding the relatively low concentrations at which estrogenic effects were observed.

DNA damage

In addition to stimulating ER-responsive tumor growth, chemicals may also cause DNA damage. Contradictory results have been published on the genotoxicity of benzophenones or metabolites. *In vitro* data from Salmonella studies (with and without bioactivation) have been predominantly negative.[13] However, BP-3 was reported to induce chromosome aberrations in CHO Chinese hamster ovary cells.[14] Furthermore, a single bacterial strain, under conditions of metabolic activation, showed weakly positive results. Robison and co-workers used the Drosophila somatic mutation and recombination test (SMART) and *in vivo* cytogenetics in rat bone marrow to define the potential for genotoxicity *in vivo* and concluded that BP-3 was not genotoxic *in vivo.*[14]

In our study, we performed an alkaline single-cell gel electrophoresis (comet) assay. Both BP-1 and BP-3 did not induce DNA damage in MCF-7 cells after 4h exposure, confirming the negative results described in earlier studies.

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7 General discussion

Estrogenic effects

Estrogenic effects of xenobiotics have been the subject of extensive toxicological research during the past decades. They play an important role in development, growth, and maintenance of female secondary sex characteristics. Furthermore, they have the ability to interfere with breast cancer development. These properties have drawn attention of both scientists and the general public to this extensive group of compounds. Estrogenic effects may be established by several pathways, most importantly via direct activation of the estrogen receptor (ER), but also via interference with the aromatase enzyme, which is responsible for estrogen biosynthesis. In this thesis, effects of xenobiotics on these two mechanisms were investigated and described.

Estrogen receptor-mediated effects

ER-mediated estrogenic effects can be assessed in various ways. First, ER-mediated cell proliferation can be studied. MCF-7 human breast carcinoma cells proliferate at a faster rate when exposed to estrogens. Cell proliferation is readily measured by various methods developed for the quantification of cell numbers. Examples are, sulforhodamine staining with subsequent spectrophotometric analysis,[1-4] counting nuclei in a Coulter Counter [5,6] or measuring mitochondrial reduction of MTT,[1-4,6,7]

Other markers for estrogenicity in MCF-7 cells are pS2 gene transcription and protein synthesis. pS2 gene transcription is regulated in an estrogen-dependent manner. The function of the pS2 protein is unknown, but the pS2 gene was originally identified as an estrogen-inducible transcript in MCF-7 cells,[8] pS2 protein levels,[4,9] as well as pS2 gene transcription [10-13] can be used as a measure of the estrogenic properties of compounds.[5]

Another possibility to identify ER-mediated effects, is to use various reporter-gene assays that have been developed to measure ER-dependent transcriptional and translational activity.[6,14-16]

Furthermore, a direct measurement of the ER-binding capacity of the compound of interest can also be performed. For this purpose, radiolabeled estradiol

is added to cells or cytosolic fractions containing ERs. The ability of a compound to displace the radiolabeled estradiol from the ER is a measure of estrogenicity.[9] A major backdraw of this method is that ER-antagonists also bind to the ER and displace radiolabeled estradiol. However, these compounds block the ER without eliciting an estrogenic effect. Therefore, these experiments do not give any functional information on the effects of the test compounds. A disadvantage of protocols using whole cells, is that they do not distinguish between displacement of ligand from the ER and other cellular processes that can interfere with this process.

In an ER-binding study using cytosolic fractions, 1 nM of E2 was shown to displace 50% of radiolabeled estradiol from the ER.[17] Stimulation of MCF-7 cell proliferation and induction of pS2 gene transcription responded with similar sensitivity towards estrogenic compounds, with EC_{50} values for E2 of about 10 pM (chapter 2 of this thesis). Effects of xenobiotics on estrogen receptor-mediated pS2 gene transcription were described in chapter 2 of this thesis. Commercially available ultraviolet (UV)-filters were tested for their ability to elicit estrogenic effects by measuring pS2 gene transcription levels in MCF-7 cells. Studies revealed that 75% or less of the skin surface is, often daily and repeatedly, treated with sunscreens during a period of 3 to 4 weeks in summer.[18] Many UV-filters are highly lipophilic and tend to bioaccumulate and reach high concentrations in fat tissue. For a number of these UV-filters this daily application is sufficient to cause bioaccumulation in the human body. Observed plasma concentrations, ranging from 0.03 to 0.9 μM,[19] fall within the range of concentrations (0.5 – 1.9 μM) causing a 50% increase of basal pS2 gene transcription in MCF-7 cells. Therefore, bioaccumulation has to be taken into account when assessing risks for various classes of compounds. Results from chapter 2 also indicate that the metabolite BP-1 is more estrogenic than parent compound BP-3, indicating that metabolism of UV-filters may in some instances lead to significant bioactivation, instead of deactivation to less estrogenic compounds. Bioactivation of the UV-filter 4-MBC has been demonstrated in rats and humans.[20] Clearly, bioactivation is a process that has to be considered when interpreting and extrapolating results from *in vitro* tests in which biotransformation does not take place.

To obtain data on individual compounds the previously described test systems, such as reporter gene assays, assays for cell proliferation, pS2 gene transcription, pS2 protein secretion and ER-binding, can be used. However, in reality humans are often exposed to more than one compound at the same time. Compounds in a mixture have the possibility to interact with each other and the estrogenic effect of a mixture can be calculated on the basis of the estrogenic effects of its individual components. When compounds interact and elicit a specific effect, and the combined effect is more than would have been expected based on individual effects, the interaction between the compounds is called synergistic. Antagonism occurs when the combined effect is less than would have been expected based on individual effects. Synergism and antagonism suggest different mechanisms of action of the different components in the mixture.

The concept of concentration-addition assumes that each component in a mixture acts via a similar mechanism of action. In this concept, each mixture-component contributes to the overall observed effect in proportion to its concentration in the mixture. In case of ER-mediated processes, this concept can be used for the prediction of mixture effects.

Usually, commercial sunscreens contain several UV-filters. As a result, humans are exposed to multiple UV-filters at the same time. We hypothesized that these UV-filters act through binding to the ER to elicit subsequent pS2 gene transcription in an additive manner. Our results that are presented in chapter 2, confirmed this hypothesis by showing additivity based on induction of pS2 gene transcription in MCF-7 cells exposed to binary mixtures of BP-1 and BP-3, as well as to a multi-component mixture consisting of BP-1, BP-3, 4-MBC and OMC.

In view of our results and observed plasma levels in humans [19] it cannot be excluded that daily exposure to sunscreen formulations may have estrogenic effects in humans. However, it should be noted that the relative potencies of the UV-filters are 5 to 6 orders of magnitude lower than that of E2 but their plasma levels are higher than those of E2.

Effects on aromatase

There are various ways to assess aromatase activity. The first method is quantification of the amount of aromatase protein by the use of aromatase antibodies. However, the antibodies that are available at present are not highly specific. Conflicting reports [21-23] on correlation between staining and biochemical activity question the validity of using aromatase protein levels as a measure of aromatase activity.

Identification and quantification of the product that is formed by aromatase can also be used as a measure of aromatase activity. Radiolabeled substrate is converted into radiolabeled product, which can then be quantified after several derivatisation steps and chromatography. An advantage of this method is that it measures aromatase activity with great confidence, although it is a laborious protocol that limits the number of samples that can be analysed in one experiment.

Furthermore, catalytic enzyme activity can be assessed by performing a ^{3}H-water release assay.[24,25] In this assay, substrate (androstenedione or testosterone) with a ^{3}H-label on the 1β-position is used. The aromatase enzyme incorporates this label in the watermolecule that is formed during the reaction. After extraction of organic compounds the amount of tritiated watermolecules, as a measure of aromatase activity, can be quantified using a liquid scintillation counter. Results obtained by this method can be influenced by other mechanisms that have the ability to incorporate the tritiated H-atom into water.

Aromatase mRNA levels can be quantified in order to assess effects of xenobiotics on aromatase gene transcription. When using promoter specific primers, one can not only study whether or not a compound has the ability to induce aromatase gene transcription, but also identify the second messenger pathway that is utilized to elicit the effect.

Aromatase inhibition

For the studies that are described in this thesis, the methods for the ^{3}H-water release assay and aromatase gene transcription were used. In chapter 4, we studied effects of $MeSO_2$-PCB metabolites on aromatase.[26] $MeSO_2$-PCB metabolites are persistent contaminants that are ubiquitously present in humans and the environment. They have been detected in human milk, adipose, liver and lung tissue. Exposure to organochlorines has been linked to possible adverse endocrine effects observed in wildlife, laboratory animals and humans,[27] some of which can be observed at tissue concentrations not much greater than in background exposure populations in industrialized countries. Human exposure to PCBs has been associated with testicular cancer and decreased sperm counts in males and may play a role in the development of breast cancer in women.[28-30]

Of the $MeSO_2$-PCB-metabolites that were studied, four compounds significantly decreased aromatase activity concentration-dependently in primary human mammary fibroblasts, as well as in H295R human adrenocortical carcinoma cells. Further experiments showed that the decrease in enzyme activity was caused by catalytic inhibition and not by a decrease of aromatase gene transcription levels. Comparison with the pharmacological aromatase inhibitor letrozole, revealed that there is a difference of 5 orders of magnitude between human body burden and pharmacologically active concentrations. Therefore, a biologically significant effect of $MeSO_2$-PCB exposure on aromatase activity is not expected.

Effects on aromatase of the frequently used pesticides epoxyconazole (EPO) and prochloraz (PRO) were also studied. Both pesticides inhibited aromatase activity completely in the rat Leydig carcinoma cell line R2C, as well as in the H295R cell line. Enzyme kinetic studies revealed that epoxyconazole inhibits aromatase activity in a competitive manner. The same was true for various other triazole compounds that were previously tested in H295R cells.[25] Imidazole-like compounds, such as imazalil, PRO and fadrozole (FAD), showed mixed-type inhibition of aromatase activity.[31-33] IC_{50} values were 1 µM for both FAD and PRO and approximately 3 µM for EPO,[33] indicating similar potencies for the clinically used second generation aromatase inhibitor FAD and the pesticides PRO and EPO.

Clinical applications of aromatase inhibitors

Aromatase inhibitors are used as pharmacological agents during treatment of estrogen-responsive breast cancer. By blocking estrogen biosynthesis, tumors are deprived from estrogens and stop growing or even regress. Aromatase inhibitors are used in both the neo-adjuvant (aims to shrink the tumor until operable) and the adjuvant setting (aims to prevent the tumor from recurring after surgery). First generation steroidal aromatase inhibitors (such as testolactone and aminoglutethimide) were mainly irreversible inihibitors of aromatase by blocking the substrate binding site. Second generation steroidal aromatase inhibitors (such as formestane and fadrozole) were modified in such a way that aromatase converts them into reactive intermediates that bind tightly and irreversibly to aromatase.[34] This type of inhibition was called mechanism-based or 'suicide' inhibition. Letrozole, anastrazole and exemestane are examples of third generation aromatase inhibitors.[35] Exemestane is a steroidal compound and binds irreversibly tot the substrate binding site. Letrozole and anastrazole are non-steroidal aromatase inhibitors, which bind reversibly to the heme-group of the aromatase enzyme instead of the substrate binding site. Miller and co-workers showed that letrozole is the most potent aromatase inhibitor in cultured fibroblasts with an IC_{50} of 0.8 nM.[36] Letrozole and anastrazole are triazoles and not surprisingly, pesticides with an imidazole or triazole molecular structure, have been shown to interact with aromatase as well (see chapter 3 of this thesis).

Aromatase inducers

In addition to aromatase inhibitors, chapter 3 also studied known pharmacological aromatase inducers. When H295R cells were exposed to 8-Br-cAMP, DEX, PMA or PGE2, aromatase activity increased concentration-dependently. Promoter I.3 and pII-specific aromatase gene transcription was elevated after exposure of H295R cells to 8-Br-cAMP, PGE2 and PMA. However, CYP19 transcript derived from promoter region I.4 was not detectable in H295R cells.

In our study, atrazine was the only pesticide capable of inducing aromatase activity about 3-fold in H295R. Additionally, natural flavonoids were also shown to

have the ability to induce aromatase activity in H295R cells. Quercetin, genistein, flavone and atrazine exert their effect on aromatase activity by increasing intracellular cAMP levels and subsequently increasing I.3- and pII- specific aromatase gene transcription.[31,37]

Direct and indirect estrogenic effects and breast cancer development

During normal breast development, interactions between epithelial and mesenchymal cells play an essential role in epithelial cell proliferation and differentiation. Stromal cells have also been suggested to play an important role in breast cancer development.[38] Paracrine interactions are important physiological processes by which neighboring cells can modulate each others activity and maintain proper tissue integrity. Expression of specific genes is highly dependent on receiving coordinated extracellular signals.[13,39]

As described previously, estrogenic effects can be elicited through direct activation of the ER, as well as through induction of aromatase activity, resulting in elevated estrogen levels which in turn activate the ER. In case of an estrogen-responsive breast tumor, paracrine interactions between cell types are thought to be established and various compounds may interfere with these processes. Cell proliferation of estrogen-responsive epithelial tumor cells can be induced by compounds that directly activate the ER. As a result of cell proliferation, these compounds cause increased secretion of factors such as IL-6sR, in turn leading to elevated aromatase gene transcription and subsequent synthesis of E2 in the surrounding fibroblasts. IL-6sR secretion is an ER-mediated event, which is completely blocked by the anti-estrogen 4-hydroxytamoxifen.[40] In addition to IL-6sR, factors such as PGE2 and IL-6 are also secreted by epithelial tumor cells when exposed to estrogenic compounds. In response to these factors, surrounding fibroblasts increase aromatase gene transcription, leading to increased aromatase enzyme levels and subsequent higher local estrogen synthesis.

DEX induces aromatase gene transcription directly via promoter region I.4,[41] but DEX also elevates IL-6sR secretion by MCF-7 cells [40,42] resulting in increased

stimulatory signals for aromatase promoter region I.4 in the adjacent fibroblasts, possibly leading to synergistic action of both mechanisms.

Development of an in vitro breast cancer model

To assess direct and indirect estrogenic properties of compounds and study paracrine interactions between a breast tumor and its surrounding tissue, we further developed an *in vitro* model for breast cancer, consisting of primary human mammary fibroblasts co-cultured with MCF-7 human mammary carcinoma cells. The validation of this model was described in chapter 5. It has long been hypothesized that paracrine interactions between breast tumor cells and surrounding fibroblasts occur. Gache and co-workers showed that primary human mammary fibroblasts, co-cultured with MCF-7 cells, stimulated MCF-7 cell proliferation.[38] Furthermore, Singh and co-workers suggested that MCF-7 cells induce aromatase activity in fibroblasts in a paracrine manner by secreting factors such as IL-6 and PGE2.[43-46] To our knowledge, this is the first *in vitro* study that describes these paracrine interactions in both directions. The strong indications that paracrine interactions occur has implications for breast cancer drug development as well as risk assessment of chemicals acting either on the ER or on aromatase.

Our observation that estrogenic effects caused by xenoestrogens, such as BPA, are potentiated in a co-culture of both MCF-7 cells and fibroblasts, requires reassessment of the endocrine disrupting potential of these xenobiotics with respect to breast cancer development and of the types of test systems used. Furthermore, compounds that stimulate aromatase expression and aromatase enzyme activity have to be taken into account when looking for potential endocrine disruptors and possible promoters of already existing breast tumors as these compounds may elicit indirect estrogenic effects resulting in tumor promotion.

The nature of the secreted factors that are responsible for the paracrine interactions between breast tumor cells and surrounding tissue should clearly be further elucidated. Drugs that specifically target secretion of these factors could be developed and added to the battery of existing drugs to treat estrogen-responsive breast cancer.

Although the *in vitro* co-culture breast cancer model described in this thesis is a more relevant test system to assess effects on breast tumor promotion than MCF-7 mono-culture, it is not suitable for high-throughput screening. It requires a steady supply of human primary tissue; furthermore, the high variability in aromatase activity observed among patients makes it difficult to interpret data in a quantitative manner. Therefore, alternate high-throughput screening tools for effects on aromatase need to be developed. In chapter 3, we compared two cell lines for their suitability as screening tools for effects on aromatase activity. R2C rat Leydig carcinoma cells were almost 10 times more sensitive towards aromatase inhibitors than H295R cells under our experimental conditions.[33] Basal aromatase activity in R2C cells is high, suggesting that one or more promoters may be fully activated, resulting in a constitutively high level of aromatase gene transcription.[33,47-51] Experiments in H295R human adrenocortical carcinoma cells showed that aromatase activity was elevated after 24h exposure to cAMP, DEX, PMA and PGE2, indicating that several promoter regions may be activated. Unfortunately, promoter region I.4 was not detectable in H295R cells. However, H295R cells were shown to be suitable for the assessment of aromatase induction as well as inhibition by various compounds.

Conclusions

- UV-filters in commercial sunscreens can elicit estrogenic effects in a mono-culture of MCF-7 cells, as well as in a co-culture system of MCF-7 cells and human mammary fibroblasts.
- The R2C rat Leydig carcinoma cell line is suitable for the detection of aromatase inhibitors.
- The H295R human adrenocortical carcinoma cell line is suitable to test for the detection of both inducers and inhibitors of aromatase activity.
- Methyl sulfonyl PCB metabolites inhibit aromatase activity in primary human mammary fibroblasts and several frequently used pesticides either induce or inhibit aromatase activity in the H295R human adrenocortical carcinoma cell line or in the R2C rat Leydig carcinoma cell line.

- Increased estrogenic effects after aromatase induction and increased aromatase gene transcription after exposure to estrogenic compounds, suggest the presence of paracrine interactions between the two cell types in our co-culture of MCF-7 cells and primary human mammary fibrobasts.
- Indirect estrogenic effects caused by induction of aromatase activity should be considered when testing for estrogenic effects of compounds.
- Increased estrogenic effects of BPA in a co-culture of MCF-7 cells and primary human mammary fibroblasts, suggest paracrine interactions between breast tumor cells and surrounding fibroblasts. These paracrine interactions can elevate estrogenic effects and should to be taken into account when performing risk assessment of endocrine disruptors.

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Samenvatting in het Nederlands

Geslachtshormonen

Het hormonale systeem is een complex systeem dat zorgt voor een fysiologisch evenwicht in het lichaam. Het bestaat uit verscheidene organen die vele verschillende hormonen produceren. Mannelijke en vrouwelijke geslachtshormonen worden geproduceerd door de bijnierschors, eierstokken en testes (teelballen). In de bijnierschors zorgen de twee binnenste lagen (zonae fasciculata en reticularis) voor de productie van geslachtshormonen, hoewel deze productie in het niet valt bij de productie in de eierstokken en de testes. In de testes, zorgen Leydig cellen voor de productie van mannelijke geslachtshormonen, zoals testosteron. In de eierstokken omringen theca cellen en granulosa cellen de follikels die de rijpende eicellen bevatten. Theca cellen produceren testosteron dat door granulosa cellen wordt omgezet in vrouwelijke geslachtshormonen, zoals oestradiol.

Deze geslachtshormonen kunnen zich via de bloedbaan door het lichaam verspreiden en in specifieke weefsels aan hormoonreceptoren binden. Eenmaal daar aangekomen kunnen de hormonen allerlei mechanismen (second messenger pathways) in het cytoplasma van de cellen beïnvloeden. Ook kunnen ze de celkern ingaan. Daar binden ze dan aan bepaalde stukken DNA waardoor afschrijving (expressie) van specifieke genen wordt gestimuleerd of geblokkeerd.

Functie en productie van oestrogenen

Oestrogenen is de verzamelnaam voor de verschillende vrouwelijke geslachtshormonen, zoals oestradiol en oestron. Oestrogenen spelen een belangrijke rol in de groei, de ontwikkeling en het functioneren van verschillende organen, zoals de baarmoeder, vagina, melkklieren, testes en de prostaat.

Aromatase (cytochroom P450 19) is het enzym dat verantwoordelijk is voor de omzetting van mannelijk (androgeen) naar vrouwelijk (oestrogeen) geslachtshormoon. In de meeste gewervelde diersoorten is aromatase gedetecteerd in de eierstokken, testes en hersenen. In mensen is het ook gevonden in placenta, bindweefsel, botten en sommige foetale weefsels. Expressie van het aromatase gen wordt per soort en per weefsel anders geregeld.

Hormoonverstoring

De wereldgezondheidsorganisatie heeft een hormoonverstorende stof gedefinieerd als ‘een lichaamsvreemde stof of mengsel dat de functie van het hormonale systeem verandert en negatieve gezondheidseffecten veroorzaakt in een intact organisme, zijn nakomelingen of subpopulaties’. Er zijn vele stoffen, zowel synthetische als natuurlijke, die het hormoonsysteem kunnen verstoren. Stoffen kunnen bijvoorbeeld aan hormoonreceptoren binden en op die manier gen expressieniveau´s veranderen. Ze kunnen ook interacteren met mechanismen (second messenger pathways) in cellen of met aromatase en op die manier een negatief effect hebben op reproductie, ontwikkeling of hormoonafhankelijke tumorgroei. In dit proefschrift zijn effecten van lichaamsvreemde stoffen op aromatase en hormoonreceptor binding onderzocht en beschreven.

Oestrogeenreceptor gemedieerde effecten

Van vele stoffen is bekend dat ze aan de receptor voor oestrogenen kunnen binden. Voorbeelden zijn bisphenol A, diethylstilbestrol (DES) en DDE. Bisphenol A wordt gebruikt als weekmaker in plastics, DES werd gebruikt als medicijn om de zwangerschap mee te bevorderen en DDE is een afbraakproduct van het veelgebruikte insecticide DDT.

Om te testen of stoffen oestrogene effecten kunnen veroorzaken worden veel *in vitro* testen uitgevoerd. Men kijkt dan of een stof aan de oestrogeenreceptor kan binden. Ook kan men onderzoeken of na blootstelling aan de stof de expressie van een specifiek, door oestrogenen gereguleerd, gen wordt verhoogd. Ook kan gekeken worden naar cellen waarvan de groei wordt gereguleerd door oestrogenen. De toename in aantal cellen wordt dan gebruikt als een maat voor oestrogene effecten van een bepaalde stof.

Naast *in vitro* testen worden ook *in vivo* testen uitgevoerd. In dit geval worden bijvoorbeeld ratten blootgesteld aan de te testen stoffen. Na verloop van tijd wordt dan gekeken naar het gewicht van bepaalde voortplantingsorganen, hormoonconcentraties in het bloed, gedrag en/of groei.

In hoofdstuk 2 van dit proefschrift is gekeken naar de oestrogene effecten van UV-filters die gebruikt worden in zonnebrandmiddelen. Menselijke borstkankercellen (MCF-7 cellen) werden blootgesteld aan deze stoffen en vervolgens werd het expressieniveau van het pS2 gen bepaald. De expressie van dit gen wordt gereguleerd door oestrogene stoffen. Vier van de geteste stoffen stimuleerden pS2 gen expressie.

Tijdens de zomermaanden wordt vaak 75% van het lichaam dagelijks ingesmeerd met zonnebrandmiddelen. Doordat de UV-filters in deze zonnebrandmiddelen goed in vet oplossen en minder goed in water (en bloed), hopen deze stoffen zich op in het lichaam. Hierdoor neemt de concentratie van deze stoffen in het lichaam toe. Daarbij zitten er vaak meerdere UV-filters in één zonnebrandmiddel. Deze stoffen binden alle aan de oestrogeenreceptor. Gemeten plasmaconcentraties zijn zó hoog dat ze in onze experimenten een toename in pS2 gen expressie van 50% zouden veroorzaken. Het kan dus niet uitgesloten worden dat dagelijkse blootstelling aan zonnebrandmiddelen oestrogene effecten kan veroorzaken in mensen.

Aromatase

Ook stoffen die de activiteit van aromatase kunnen beïnvloeden, hebben de laatste tijd aandacht gekregen. Immers, als een stof niet aan de oestrogeenreceptor bindt, kan het wel zijn dat deze stof de aromatase activiteit verhoogt. Aangezien aromatase een sleutelrol speelt in de productie van oestrogenen, zou blootstelling aan de stof een verhoging van de oestrogeenproductie tot gevolg hebben. De daarop volgende hogere concentraties van oestrogenen in het bloed kunnen leiden tot versterkte oestrogene effecten. In dat geval heeft de stof dus zelf niet direct een oestrogeen effect veroorzaakt, maar wel indirect.

Van verschillende stoffen is bekend dat ze aromatase activiteit beïnvloeden. Bestrijdingsmiddelen zoals imizalil en prochloraz verlagen aromatase activiteit. Maar atrazine en vinclozoline kunnen aromatase activiteit juist verhogen.

Omzettingsproducten (metabolieten) van PCB's zijn overal in het milieu te vinden. Ze zijn gevonden in moedermelk, vet, lever en longen van wilde dieren en mensen. In hoofdstuk 4 worden effecten van deze PCB-metabolieten op aromatase beschreven. Uit de experimenten bleek dat vier van de geteste PCB-metabolieten een remmende werking op aromatase hadden. Onderzoek naar het mechanisme hierachter wees uit dat deze stoffen het enzym blokkeren zonder de gen expressie van aromatase af te remmen. Vergeleken met de aromatase remmer letrozole, die gebruikt wordt bij de behandeling van borstkanker, zijn de PCB-metabolieten erg zwak. Er was een 100.000 keer hogere concentratie nodig van de PCB-metabolieten dan van letrozole om hetzelfde

effect te bewerkstelligen. Blootstelling aan deze PCB-metabolieten leidt dus hoogst waarschijnlijk niet tot een biologisch relevant effect op aromatase activiteit in de mens.

Veranderingen in aromatase activiteit kunnen dus leiden tot veranderingen in oestrogeenniveaus in het bloed. Lagere oestrogeenconcentraties kunnen leiden tot verminderde vruchtbaarheid en botontkalking. Verhoogde oestrogeenniveaus worden geassocieerd met een verhoogde kans op de ontwikkeling van borstkanker.

Borstkanker

Kwaadaardige borsttumoren zijn de meest voorkomende tumoren bij vrouwen. Ongeveer tweederde van deze tumoren bevatten oestrogeenreceptoren waardoor hun groei afhankelijk is van oestrogenen. Niet alleen hormonen maar ook lichaamsvreemde stoffen, zoals bisphenol A en DES, kunnen de hormoonreceptoren activeren en dus tumorgroei stimuleren. In een borsttumor worden de tumorcellen omringd door bindweefsel. Deze twee celtypen communiceren met elkaar door middel van het uitscheiden van allerlei stoffen. Het bindweefsel (fibroblasten) bevat het aromatase enzym, waardoor zij oestrogenen maken. Deze oestrogenen diffunderen naar de tumorcellen waar ze kunnen binden aan de oestrogeenreceptoren. Door deze binding gaan de tumorcellen delen en tijdens deze deling scheiden ze stoffen uit die kunnen diffunderen naar de bindweefsel cellen. Deze stoffen kunnen op hun beurt aromatase activiteit in de bindweefsel cellen stimuleren. Op deze manier ontstaat er een positieve terugkoppeling tussen de twee celtypes.

Directe en indirecte oestrogene effecten en de ontwikkeling van borstkanker

Door de positieve terugkoppeling tussen de twee celtypes (bindweefselcellen en tumorcellen) in borstkanker kunnen stoffen zowel directe als indirecte effecten bewerkstelligen. Een oestrogene stof, zoals oestradiol, bindt aan de oestrogeenreceptor en veroorzaakt op die manier een direct oestrogeen effect in de tumor cel. Als gevolg daarvan scheiden de tumorcellen stoffen uit die aromatase stimuleren in het omliggende bindweefsel. Oestradiol heeft dan dus een indirect effect op aromatase. Voor een stof die aromatase stimuleert, zoals dexamethason, geldt een soortgelijk mechanisme. Dexamethason stimuleert aromatase in het bindweefsel op een directe manier. Hierdoor worden oestrogenen door deze cellen geproduceerd. Deze diffunderen naar de

tumorcellen die vervolgens gaan groeien. Dexamethason heeft dus een indirect oestrogeen effect op de tumorcellen.

Ontwikkeling van een *in vitro* model voor borstkanker

Om directe en indirecte oestrogene effecten te onderzoeken, hebben we een *in vitro* borstkanker model ontwikkeld. Menselijke borsttumorcellen (MCF-7 cellen) zijn samen gekweekt (in een co-culture) met menselijke bindweefsel cellen. Door ze in één ruimte te kweken, was het mogelijk om de communicatie tussen de cellen te bewerkstelligen. In hoofdstuk 5 is de ontwikkeling en de validatie van dit model beschreven. Het effect van bepaalde oestrogene stoffen werd versterkt door het toevoegen van testosteron aan het kweekmedium. Het testosteron diende als substraat voor aromatase in het bindweefsel en werd dus omgezet in oestradiol. Dit extra oestradiol zorgde voor een versterkt oestrogeen effect in de tumorcellen. Wanneer we de co-culture blootstelden aan dexamethason zagen we een direct effect op aromatase gen expressie in het bindweefsel. Als dan ook testosteron aan het medium was toegevoegd zagen we ook een effect op pS2 gen expressie in de borsttumor cellen. Deze resultaten suggereren dat in dit model de positieve terugkoppeling tot stand is gekomen. De indicatie dat deze positieve terugkoppeling daadwerkelijk aanwezig is in het menselijke lichaam kan betekenen dat hormoonverstorende eigenschappen van stoffen opnieuw gekarakteriseerd moeten worden. De testen die tot nu toe zijn uitgevoerd hielden namelijk geen rekening met deze positieve terugkoppeling. Ook moeten stoffen gescreend worden op hun potentiële capaciteit om aromatase te beïnvloeden. Als stoffen geen invloed hebben op oestrogeenreceptor gemedieerde processen, maar wel op de activiteit van het aromatase enzym, kunnen ze dus alsnog een (indirect) oestrogeen effect veroorzaken. Voor het testen op effecten op aromatase moeten testsystemen ontwikkeld worden. Hoofdstuk 3 beschrijft twee cellijnen, een menselijke bijnierschorscellijn (H295R) en een testes cellijn van een rat (R2C), die beide geschikt zijn voor het testen van effecten van verschillende stoffen op aromatase.

Conclusies

- UV-filters die in commerciële zonnebrandmiddelen te vinden zijn kunnen oestrogene effecten veroorzaken in borsttumorcellen en in de co-culture van borsttumorcellen en bindweefsel.
- De ratten cellijn R2C is bruikbaar voor het testen van remmende effecten op aromatase.
- De menselijke cellijn H295R is bruikbaar voor het testen van stimulerende en remmende effecten op aromatase.
- PCB-metabolieten remmen aromatase activiteit in menselijk bindweefsel en verschillende bestrijdingsmiddelen stimuleren of remmen aromatase activiteit in ratten testescellen (R2C) en menselijke bijnierschorskankercellen (H295R).
- Stimulatie van aromatase activiteit in bindweefselcellen veroorzaakt versterkte oestrogene effecten in borsttumorcellen. Blootstelling aan oestrogene stoffen veroorzaakt een verhoogde aromatase genexpressie in bindweefselcellen. Deze twee observaties suggereren een positieve terugkoppeling tussen deze twee celtypes in de co-culture van borsttumorcellen en bindweefsel.
- Indirecte oestrogene effecten als gevolg van stimulatie van aromatase zouden in acht moeten worden genomen bij de risico-evaluatie van chemicaliën.
- De positieve terugkoppeling tussen borstkankercellen en bindweefsel kan oestrogene effecten van stoffen versterken. Daarom moet er rekening gehouden worden met deze positieve terugkoppeling bij de risico-evaluatie van chemicaliën.

Curriculum Vitae

Marjoke Heneweer werd op 9 september 1975 geboren in Amersfoort. In 1993 behaalde zij haar VWO diploma aan het Meridiaan College, vestiging Het nieuwe Eemland in Amersfoort. Na een jaar werken aan de Ecole d'Humanité in Zwitserland, startte zij in 1994 met de studie Psychologie aan de Universiteit Utrecht. Na het eerste jaar en het behalen van de propedeuse besloot zij in september 1995 te beginnen met de studie Biologie aan dezelfde universiteit. Tijdens de studie werden er stages gelopen bij het Research Institute for Toxicology (RITOX) met als begeleiders Dr. J. Thomas Sanderson en Dr. Robert. J. Letcher en bij de vakgroep Aquatic Toxicology van Michigan State University, onder begeleiding van Dr. Trine Celius en Prof. Dr. John. P. Giesy. Aansluitend op het behalen van het doctoraaldiploma is gestart met het promotie onderzoek dat beschreven is in dit proefschrift. Het promotie onderzoek ging over aromatase en oestrogeenreceptor gemedieerde processen in de ontwikkeling van borstkanker. Het onderzoek vond plaats op het Institute for Risk Assessment Sciences (IRAS), vakgroep Biochemische Toxicologie en de begeleiding was in handen van Dr. J. Thomas Sanderson en Prof. Dr. Martin van den Berg.

Marjoke Heneweer was born on September 9th, 1975 in Amersfoort, The Netherlands. In 1993 she graduated from Meridiaan College, Het nieuwe Eemland in Amersfoort. She worked at the Ecole d'Humanité in Switzerland for one year before she started studying Psychology at Utrecht University (Utrecht, The Netherlands). After one year she decided to change to the Biology program at the same university. During this program she worked at the Research Institute for Toxicology (RITOX) under supervision of Dr. J. Thomas Sanderson and Dr. Robert. J. Letcher. Furthermore, she worked with Dr. Trine Celius and Prof. Dr. John P. Giesy within the Aquatic Toxicology department at Michigan State University (East Lansing, MI, USA). After graduation, she started her Ph.D.-project that was described in this thesis. Supervisors of this project were Dr. J. Thomas Sanderson and Prof. Dr. Martin van den Berg. The project was conducted at the Institute for Risk Assessment Sciences (IRAS) at Utrecht University and focussed on aromatase and estrogen receptor-mediated processes in breast cancer development.

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