In vivo relevance of in vitro detected estrogenic effects of food associated compounds 2008 MGR ter Veld

In vivo relevance of in vitro detected estrogenic effects of food associated compounds

Marcel ter Veld

Stellingen

1. De *in vivo* effecten van stoffen die *in vitro* estrogeen zijn bevonden worden overschat als geen rekening gehouden wordt met mogelijk geringe biobeschikbaarheid.
Dit proefschrift

2. Van nieuwe weekmakers gebaseerd op sorbitol, zijn geen estrogene effecten te verwachten wat ze veelbelovende vervangers maakt voor bestaande weekmakers zoals ftalaten.
Dit proefschrift

3. De conclusie; "Chronic exposure to toxic environmental agents, …, lead to a decline in birth rate": is niet te weerleggen louter op basis van onderzoek naar de effecten van stoffen op het reproduktiesysteem 'an sich'.
Rasier *et al.* (2006), Molecular and Cellular Endocrinology, 254: 187

4. Onderzoekers die stellen dat gefistuleerde knaagdieren het beste model zijn voor onderzoek naar Bulimia Nervosa, staan ver van de werkelijkheid.
Casper *et al.* (2008), Psychopharmacology, 199: 313

5. De tv commercial waarin de suggestie wordt gewekt dat dierproeven uitgevoerd worden met een willekeurige, onbekende stof op een willekeurig huisdier van een willekeurige voorbijganger, onder niet gecontroleerde omstandigheden, getuigt van geringe kennis omtrent de uitgebreide ethiek bij onderzoekers en regelgeving van de overheid rondom proefdiergebruik in Nederland.
Reclame proefdiervrij, 2005/6

6. De krantenkop: "Deo Beckham kan sperma aantasten" getuigt van sensatiejournalistiek.
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In vivo relevance of in vitro detected estrogenic effects of food associated compounds

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In vivo relevance of in vitro detected estrogenic effects of food associated compounds

Marcel Gerben Rudolph ter Veld

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“Kunt u me vertellen welke kant ik op moet?”
“Dat hangt er heel erg vanaf welke kant je op wilt.” Zei de kat.
“Dat kan me niet zoveel schelen.” Zei Alice.
“Dan doet het er niet toe welke kant je opgaat.” Zei de kat.
Uit: De avonturen van Alice in Wonderland





Abstract

The aim of the present thesis was to study the *in vivo* relevance of *in vitro* detected estrogenic effects of food associated compounds, with emphasis on prenatal exposure. The estrogenic potency of 21 food packaging-associated compounds was studied in ERα or ERβ transfected U2-OS (human osteoblasts devoid of endogenous estrogen receptors) cell lines. Six plasticizers and three anti-oxidants were slightly estrogenic in the ERα cells. BPA, NP, tris (2-ethylhexyl) trimellitate (TEHTM), propyl gallate and butylated hydroxy anisole (BHA) were estrogenic both in ERα and ERβ cells. These compounds appeared to be more estrogenic relative to estradiol (E_2) in ERβ than in ERα cells. To study the *in vivo* relevance of these effects, *in vivo* biomarker-responses of BPA, NP, DEHA, DEHP, DIHP, *p,p'*-DDE and quercetin were studied in ER-Luc male mice. Of these seven compounds, BPA, DEHP and quercetin induced estrogenic effects after a single oral dosage at exposure levels 10-10^4 times higher than the established Tolerable Daily Intakes (TDI's). It remains to be seen whether these margins are sufficiently high to allow the conclusion that these compounds are unlikely to represent a human health risk. As exposure to estrogenic compounds during developmental stages could be an important risk factor in developing hormone dependent cancers later in life, these seven compounds were studied in pregnant ER-Luc mice as well. After oral exposure of the mother animal the compounds were unable to significantly induce luc-activity in any of the tissues including fetuses. Unexpectedly however, NP, BPA and DIHP significantly lowered the placental luc-activity. The results indicate that at the current levels of exposure to food associated estrogenic compounds, direct estrogenic effects in the fetus are not expected. The mechanism and consequences of the significant luc-reduction in the placenta should be investigated to a further extent to elucidate its possible significance. Because of the absence of significant luc-induction in the fetuses, the fate and distribution of radioactively labeled E_2, NP and *p,p*'-DDE were studied in pregnant C57black mice in order to investigate whether the compounds can actually reach the fetuses. E_2 did not reach the fetus at a level above the detection limit, NP and *p,p*'-DDE levels were above the detection limit in fetuses exposed via the mother from GD8-16. Levels of E_2 and NP detected in the placenta were significantly higher than those in the fetuses, up to five fold for E_2 and three fold for NP, possibly due to prevention of transport of the compounds to the fetuses by placental binding proteins.

Based on the findings of the present thesis it was concluded that in spite of the *in vitro* estrogenicity of various food-born estrogens, *in vivo* estrogenic effects are not likely expected as estrogenicity *in vivo* is shown at levels 10-10^4 fold higher than the TDI's for humans and placental binding proteins may reduce fetal exposure. However, the estrogenic compounds were given as a single acute dose, whereas TDI values and also risks associated with dietary exposure can be expected to result from chronic combined exposure. Therefore, it remains to be seen whether the estimated margins are sufficiently high. Future experiments are therefore needed that focus on long term and combined exposure. Another factor that remains to be solved relates to the fact that only in *in vitro* assays the relative ability of compounds to differentially activate ERα or ERβ can be quantified.

All together the results of the present thesis reveal that the extent at which *in vitro* estrogenic effects may result in *in vivo* estrogenicity may vary with the compound under study and should be evaluated on a case by case basis taking differences in absorption, distribution, metabolism and excretion, the intrinsic estrogenic potency of chemicals for either the ERα or ERβ and the differential levels of expression of these receptors in different tissues *in vivo* into account.

Chapter 1:

General Introduction

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1

Background on endocrine disruption and aim of the thesis

The book "Our stolen future" increased the public awareness of compounds mimicking hormones and their effects (*1*). Why are the effects of hormone mimicking compounds, so called endocrine disruptors, so important? An answer to this question might be found in the fact that the endogenous female hormone estradiol (E_2) has positive but also negative effects in men and women. In men, excessive estrogen exposure during fetal development is known to cause breast development (gynaecomastia) (*2*). In postmenopausal women, estrogens are known to increase the incidence of breast cancer development (*3*). High estrogen levels in the blood of young Puerto Rican girls caused early menarche, which is a known risk factor in developing breast cancer (*4*).

Exposure to high levels of estrogens are therefore of special interest, especially exposure to high levels of exogenous compound, the endocrine disruptors, which are mimicking the endogenous hormone. An endocrine disruptor can be defined as follows: "An exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (*5*), or slightly different: "An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function." (*6*).

The link between endocrine disruptors, such as DDT, nonylphenol, bisphenol A, phthalates and hormone dependent cancers has been debated for years (*7-18*). In general, there is an increasing scientific interest in possible adverse health effects caused by human exposure to exogenous estrogenic compounds via the food. Estrogenic compounds can be present in food as contaminants (such as DDT), phytoestrogens (eg genistein and quercetin) or, amongst others, compounds that leak from packaging material (phthalates, bisphenol A (BPA) and nonylphenol (NP)). To test the estrogenicity of compounds, several in vitro and in vivo assays have been developed. In vitro the estrogenicity can be determined via assays based on for instance binding to the endogenous estrogen receptors (ERα or ERβ). As in vitro systems lack the major absorption, distribution, metabolism and excretion routes and the general physiological processes of the whole organism, the estrogenicity of compounds should be examined in vivo as well before drawing conclusions.

Therefore the aim of the present thesis was to study the in vivo relevance of in vitro detected estrogenic effects of food associated compounds.

Mechanisms of estrogenic action and possible estrogen disruption by pseudoestrogens

The female steroid hormone estradiol (E_2) is essential for several physiological mechanisms, amongst which are the development of secondary sex characteristics, induction of mammary gland development, mucus secretion of the cervix, regulation of the female body distribution of fat, bone turnover and others (*2,19*). An overview of E_2 dependent physiological processes in humans and rodents is given in Table 1.

1

Table 1: E_2 plays an important role in several physiological mechanisms during multiple life stages in humans as well as rodents, the most important life stages are presented in tabular format below (EW = Embryonic Week, GD = Gestation Day), data based on references *20* and *21*.

Events in Hormonal Life	Humans	Rodents (R=Rat, M=Mouse)
Preparation functional lactation	Pregnancy	Pregnancy
Milk streak event	EW4-6	GD10-11(M)
Mammary epithelial bud formation	EW10-13	GD12-14 (M), GD14-16 (R)
Female nipple and areola formation	EW12-16	GD18 (M), GD20 (R)
Branching and canalization of epithelium	EW20-32	GD16 to birth (M), GD18 to birth (R)
CNS sexual differentiation	Before birth	Before birth
Secretion is possible	EW32-40	At birth with hormones
Isometric development of ducts	Birth to puberty	Birth to puberty
Terminal end buds present (peripubertal)	8-13 yr old girls	23-60 days (R)
Vaginal opening	-	35 days (R)
Estrous cycle	-	40 days (R)
Formation of lobular units	EW32-40 / Within 1-2yr 1st cycle	Puberty into adulthood
Breast development	~11 yr	-
Menarche	~13 yr	-
Ovulatory cycles	~16 yr	-

E_2 levels are regulated by the hypothalamic release of Gonadotropin-Releasing Hormone (GnRH) which causes the release of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) from the pituitary, two hormones regulating the E_2 metabolism via the gonads (*2, 22*).

Upon entering the cell, E_2 has to bind to an estrogen receptor (ER), to exert effects. Two types of ER's are known; since the discovery of an ER with slightly different binding affinities for estrogens, the former estrogen receptor was named ERα and the latter ERβ (*23*). The mechanism of action proceeding by receptor binding, receptor activation and subsequent induction of mRNA, proteins and physiological effects is schematically presented in Figure 1. This illustrates that estrogens exert their estrogenicity via the estrogen receptors.

Current knowledge about human exposure to estrogenic compounds and possible effects

As stated above, there is evidence but also scientific debate regarding a role for endocrine disruptors in the induction of hormone-dependent cancers. Examples of reported effects include the relation of hormone replacement therapy and breast cancer in post-menopausal women (*25,26*). On the other hand oral contraceptives might provide protection against endometrial and ovarian cancer, whereas they may increase the risk on breast cancer (*27*). Furthermore, high doses of estrogens have been reported to cause hormone-dependent breast cancer regression in post-menopausal women (*28*).

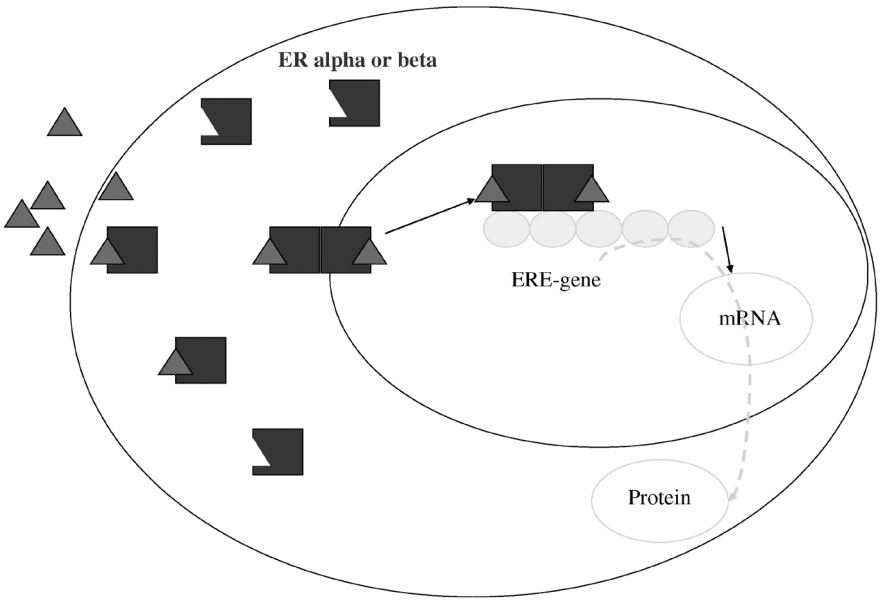

Figure 1: An estrogen (triangles) enters the target cell, binds to the estrogen receptor (ER)α or ERβ (squares). Upon dimerization of ERα or ERβ, and/or formation of ERα-ERβ dimers (*24*), these dimers will be translocated into the nucleus. There they bind to estrogen responsive elements (ERE) on the DNA present in the cell. These EREs are able to initiate transcription of genes present in the DNA, thereby resulting in mRNA synthesis and translation and production of proteins (for example vitellogenin) that cause the ultimate physiological effect.

Several papers showed an association of estrogen exposure and a general decline in semen quality (*29,30,31,32*). Besides a decline in semen quality, increases in testicular cancer, cryptorchidism (failure of testicular descent) and hypospadias (incorrect urethral opening), have been linked to exposure to environmental chemicals, such as phthalates (*32*). Puerto Rican girls with premature breast development, showed high levels of di-ester phthalates in 68% of the plasma samples. Plasma di-ester phthalate levels in these girls amounted to levels that were approximately six times higher compared to those in control samples (*33*), though phthalates are normally rapidly metabolized to their mono-esters in humans as reviewed by Latini (*33*).
A direct link of testicular germ cell cancer and exposure to high amounts of estrogens could not be established, neither be denied, in a case-control study that was relatively small (*34*). The same was concluded for estrogen-related cancers in women (*35*). Interestingly, internal (plasma) levels of estrogenic compounds are often not exactly known (*36*), and a direct causal link between exposure to estrogens and an increase in hormone-dependent cancers is difficult to prove as exposure to anti-estrogens has been increasing as well (*37,38*).
Interestingly, phytoestrogens (natural 'endocrine disruptors' present in fruits, vegetables and soy food) might protect against cancer (*39-41*).

1

Exposure and presence of estrogenic compounds in human samples

Exposure to estrogenic compounds during developmental stages is suggested to be a very important risk factor in developing several hormone dependent cancers (*9, 42-47*). Human fetal exposure to several food associated estrogenic compounds such as DDT, genistein, BPA and phthalates, has been studied thoroughly. In human amniotic fluid samples, the DDT metabolite, *p,p'*-DDE, was found up to 15 ng/mL in 25% of the 175 amnion fluid samples tested, and dietary phytoestrogens such as genistein were found in 96.2% of the 175 tested amniotic fluid samples in concentrations up to 1 ng/mL (*48*). BPA has been found in fetal plasma with a median level of 2.3 ng/mL, and median levels were 3.1 ng/mL in maternal plasma (*49*). The same ranges for BPA levels in fetal and maternal plasma were reported by Ikezuki *et al.* (*50*). Phthalates have been measured at median levels of 7.3 ng mono-ethylhexyl phthalate and 211 ng mono-ethyl phthalate per mL human urine (*51*). A slightly higher geometric mean of 345 ng mono-ethyl phthalate per mL human urine was reported as well (*52*).

Estrogenicity of food associated compounds in vitro

Some food associated estrogenic compounds were examined for their estrogenicity. As the food associated estrogenic compounds might exert their effects via the ER, several in vitro ER-based assays have been applied to measure the in vitro estrogenic potential of food associated estrogens. These in vitro assays are often based on a human cell line transfected with human estrogen receptor(s) and a reporter gene, usually luciferase, under control of estrogen responsive elements. Data reported in the literature for the in vitro estrogenic activity of various food associated estrogens are summarized in Table 2. It is remarkable that there appear to be huge (up to 10,000) differences for the observed estrogenic potency of specific compounds, when using the same assays in different laboratories and especially the study of Andersen et al. (*53*) reports deviating data.

Interestingly, it has been suggested that ERβ modulates the estrogenic effects of the ERα by forming heterodimers with ERα, thereby reducing the ERα-mediated response resulting for example in inhibition of ERα-mediated proliferation of breast cancer cells in vitro (*72-75*). The inability to obtain a full-length cDNA of the ERβ without mutation from mRNA isolated from the human T47D breast cancer and Caco-2 colon cancer cells (*76*) further supports the idea that ERβ activation may be beneficial and modulate possible adverse ERα mediated estrogenic effects. Given the differential biological responses upon ERα or ERβ activation, it is of importance in the study of estrogen-disrupting compounds to characterize their interaction with both receptors separately (*77*). As is shown in Table 2, information on the actual ER receptor activated in a specific in vitro estrogen assay, ERα or ERβ, is often not provided and also not characterized by the respective assays. Furthermore as already outlined above, these in vitro data need in vivo validation of the estrogenic effect reported for these food associated estrogenic compounds.

Aim, approach and outline of thesis

The aim of the present thesis was to study the in vivo relevance of in vitro detected estrogenic effects of food associated compounds, with emphasis on prenatal exposure and to characterize the type of estrogen receptor actually activated by these compounds. Emphasis on prenatal exposure was included in these studies because exposure to estrogenic compounds during developmental stages is suggested to be a very important risk factor in developing several hormone dependent cancers (*9, 42-47*). Furthermore results regarding either ERα or ERβ specific activation by the food associated estrogenic compounds are often lacking (shown in Table 2). Chapter 1 presents the background and aim of the thesis. To elucidate the estrogenic potency of the selected food associated estrogenic compounds towards the two individual ER receptors ERα or ERβ, in Chapter 2 of this thesis twenty different food associated compounds were tested in two human osteoblastic (U2-OS) cell lines. The two transfected cell lines stably express either ERα or ERβ in addition to 3xERE-tata-Luc as a reporter gene. The U2-OS cell line is devoid of endogenous hormone receptors, which prevents cross-talk between receptors. These studies established the *in vitro* estrogenic potency of the various selected food associated model compounds towards the two estrogen receptors.

The translation of *in vitro* estrogenicity to *in vivo* estrogenicity is very important as food associated estrogenic compounds may show very different absorption, distribution, metabolism and excretion upon *in vivo* exposure as compared to the *in vitro* situation. Therefore, the *in vitro* detected estrogenic intrinsic capacity of a food associated compound requires *in vivo* validation. In the present thesis to this end we used an ER-Luc mice containing a 3xERE-tata-Luc insulated reporter gene construct, which is able to reveal the *in vivo* activation of endogenous estrogen receptors in the various tissues. In previous studies these ER-Luc mice have been shown to be responsive to and suitable to detect *in vivo* estrogen activity. For example ER-Luc male mice showed to be responsive to DES, EP and E_2, with DES, EP and E_2 significantly inducing ER-mediated luciferase activity in various tissues after 8 h exposure at a dose of 0.1 mg DES/kg bw, 1 mg EP/kg bw or after 4 hours of exposure to 1 mg E_2/kg bw. From 0-24 h the maximum induction was reached at 8 h for E_2 and DES and at 24 h for EP (*78*). In fetuses of maternal exposed ER-Luc mice, DES and EP resulted in maximum luciferase induction at 1 mg DES/kg bw and 10 mg EP/kg bw after 24 hours exposure (*79*). Luc activity was significantly induced in fetuses 8 h after 1 and 10 mg BPA/kg bw exposure in pregnant female ER-Luc mice, but this effect was absent after 24 h (*79*). In the present thesis these ER-Luc mice were used to investigate the *in vivo* estrogenic potency of a series of food associated compounds shown to be estrogenic in *in vitro* assays. In Chapter 3 the estrogenic potency of food associated estrogenic compounds shown to be estrogenic *in vitro* in Chapter 2 was studied *in vivo* in estrogen receptor luciferase reporter gene (ER-Luc) male mice. To examine the differences in estrogenic potential of the food associated compounds, two administration routes were used. Oral exposure was used to resemble human exposure, whereas IP exposure was used to be able to compare the data from literature and to represent a worst case exposure scenario.

Table 2: Overview of data reported in the literature for the estrogenic activity of suspected food associated estrogenic compounds when tested *in vitro*. Human cell lines used in these assays are T47D cells (ER-CALUX assay), 293HEK cells, HGELN cells, all transfected with a luciferase reporter gene under control of ERE's which are activated upon binding of an estrogen to the ER. Cell lines used in the cell proliferation assays are MCF-7 cells (E-screen) and MVLN cells. The yeast cells used were generally transfected with a reporter gene under control of ERE's and human estrogen receptor(s).

Compound	Assay	EC_{50} (nM)	EEF	Reference
BPA	ER-CALUX	770	$7.7 \cdot 10^{-6}$	*54*
BPA	E-Screen	410	$1.1 \cdot 10^{-5}$	*55*
BPA	E-Screen	200	$2.5 \cdot 10^{-5}$	*56*
BPA	MVLN cells	200	$2.5 \cdot 10^{-5}$	*56*
BPA	HGELN cells	210	$1.9 \cdot 10^{-4}$	*56*
BPA	Transfected HepG2 cells	64 (α); 89 (β)	-	*57*
BPA	Yeast	3400	$6.6 \cdot 10^{-5}$	*58*
BPA	Yeast two-hybrid assay		$3.1 \cdot 10^{-5}$ (based on REC_{10})	*59*
BPA	Yeast two-hybrid assay		$1 \cdot 10^{-4}$ (based on REC_{10})	*60*
BPA	Comp. binding with ERα	15000	$2.3 \cdot 10^{-4}$	*56*
BPA	Comp. binding with ERβ	25000	$2.6 \cdot 10^{-3}$	*56*
BPA	E-Screen	20000 -70000	$1 \cdot 10^{-5}$ - $5 \cdot 10^{-7}$	*53*
BPA	YES	100-5000	$4 \cdot 10^{-5}$ - $8 \cdot 10^{-5}$	*53*
BPA	MCF-7	-	-	*61*
BPA	Transfected MCF-7 cells	71000 (α) or 45000 (β)	$7.5 \cdot 10^{-5}$ (α) or $1.8 \cdot 10^{-4}$ (β)	*62*
NP	ER-CALUX	260	$2.3 \cdot 10^{-5}$	*54; 63*
NP	293 HEK cells with either ERα or ERβ	110 (α) or 145 (β)	$8.2 \cdot 10^{-5}$ (α) or $5.5 \cdot 10^{-4}$ (β)	*63*
NP	E-Screen	400	$1.3 \cdot 10^{-5}$	*56*
NP	MVLN cells	400	$1.3 \cdot 10^{-5}$	*56*
NP	MVLN cells	463	$3.2 \cdot 10^{-5}$	*64*
NP	HGELN cells	500	$8 \cdot 10^{-5}$	*56*

Compound	Assay	EC_{50} (nM)	EEF	Reference
NP	Comp. binding with ERα	20000	$1.75 \cdot 10^{-4}$	*56*
NP	Comp. binding with ERβ	30000	$2.3 \cdot 10^{-3}$	*56*
NP	Yeast	1100	$2.1 \cdot 10^{-4}$	*58*
NP	Yeast two-hybrid assay		$7.4 \cdot 10^{-4}$ (based on REC_{10})	*59*
NP	Yeast two-hybrid assay		$1.5 \cdot 10^{-3}$ (based on REC_{10})	*60*
NP	YES	290000	$7.2 \cdot 10^{-7}$	*65*
NP	MCF-7	-	-	*65*
NP	E-Screen	100 – 8000	$7 \cdot 10^{-7}$ - $8 \cdot 10^{-8}$	*53*
NP	YES	-	-	*53*
p,p'-DDE	Transfected HepG2 cells	-	-	*57*
p,p'-DDE	Yeast and MCF-7 cells	-	-	*66*
p,p'-DDE	LUMI-cell	6740	$2.3 \cdot 10^{-6}$	*67*
p,p'-DDE	E-Screen	300 – 2000	$1 \cdot 10^{-7}$ - $1 \cdot 10^{-9}$	*53*
p,p'-DDE	YES	-	-	*53*
DEHP	Comp binding ER	-	-	*68*
DEHP	Transfected MCF-7 reporter gene cells	-	-	*68*
DEHP	ER-CALUX	>30000	$<2 \cdot 10^{-7}$	*63*
DEHP	293 HEK cells with either ERα or ERβ	>30000 (α and β)	$<3 \cdot 10^{-7}$ (α and β)	*63*
DEHP	MCF-7 cell proliferation assay	Slight increase		*69*
DEHP	Yeast two-hybrid assay		$<1.5 \cdot 10^{-7}$ (based on REC_{10})	*60*
DEHP	Rec yeast screen	-	-	*70*
DEHP	MCF-7 cells	Slight significant increase	-	*71*
DIHP	Competitive binding to the ER	-	-	*68*
DIHP	Transfected MCF-7 reporter gene cells	-	-	*68*
DEHA	Yeast two-hybrid assay		$<3 \cdot 10^{-7}$ (based on REC_{10})	*60*
DEHA	MCF-7 cells	-	-	*71*

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Due to the possibly significance of exposure to food associated estrogenic compounds during fetal development, the effects of maternal exposure to food associated estrogenic compounds and induction of ER mediated luciferase activity in fetuses of pregnant ER-Luc mice as well as the transfer of ^{14}C labeled food associated estrogenic compound to the fetus were also investigated in this thesis. To study the estrogenicity towards fetuses upon maternal exposure to food associated estrogenic compounds, in Chapter 4 the food associated estrogenic compounds were given to pregnant ER-Luc mice, using oral and IP exposure. The aim of Chapter 4 was to investigate whether *in vivo* estrogenic effects during critical fetal life stages can be expected upon maternal oral exposure to food associated estrogenic compounds.
As little estrogenicity was observed in Chapter 4 in the fetus after oral exposure of pregnant female ER-Luc mice to the food associated estrogenic compounds and a possible mechanism underlying this absence of effect may be related to the inability of these compounds to reach the fetus, the distribution within the body and transport to the fetus of three radioactive labeled food associated estrogenic compounds, E_2, NP and *p,p'*-DDE was studied in pregnant C57black mice in Chapter 5.
Together the results should provide insight in the *in vivo* relevance of *in vitro* detected estrogenic effects of food associated compounds, with emphasis on prenatal exposure.
Finally in Chapter 6 a general overview of performed studies is given and critically analyzed, and recommendations for future research are given.

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Chapter 2:

Estrogenic potency of food-packaging-associated plasticizers and antioxidants as detected in ERα and ERβ reporter gene cell lines.

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2

Abstract

This study presents the estrogenic potency of 21 food-packaging-associated compounds determined for the first time, using two transfected U2-OS (human osteoblasts devoid of endogenous estrogen receptors) estrogen receptor (ER)α and ERβ cell lines. Six plasticizers and three antioxidants were slightly estrogenic in the ERα cells. The model compounds bisphenol A and nonylphenol, one plasticizer [tris (2-ethylhexyl) trimellitate (TEHTM)], and two antioxidants (propyl gallate and butylated hydroxyanisole) were estrogenic in both ERα and ERβ cells. Compared to estradiol (E_2), these compounds appeared to be relatively more estrogenic in the ERβ cells than in the ERα cells. Three sorbitol-based plasticizers activated neither ERα nor ERβ and may be good replacements of existing plasticizers. All responses were additive with the response of E_2. This indicates that they may contribute to the total effects of the pool of estrogenic compounds humans are exposed to. The estrogenic potencies of these compounds, together with the suggested beneficial effect of ERβ-mediated responses and adverse ERα-mediated effects, support the importance of detecting characteristics for ERα and ERβ response separately in independent models, as done in the present study.

2

Introduction

Incidences of hormone-related cancers, especially breast cancer, are increasing worldwide (*1*). In The Netherlands, age-specific breast cancer incidence increased by 23% in the period from 1989 to 2000 (*2*). In addition to some known risk factors such as radiation exposure (*3*), genetic predisposition (*4*), alcohol intake (*5*), and a higher fat intake (in animal studies) (*6*), estrogenic compounds, such as natural estrogens and pseudo-estrogens, are suspected of increasing a woman's risk of developing breast cancer (*7*). Pseudo-estrogens are all estrogenic active compounds that are not naturally present in the female body. *In vitro* some of these pseudo-estrogens (genistein and quercetin) cause cell proliferation of breast cancer cells via interaction with the estrogen receptor (*8*).

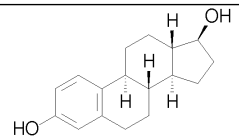
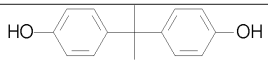
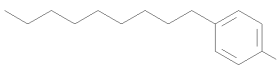
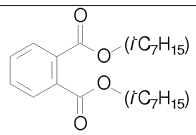
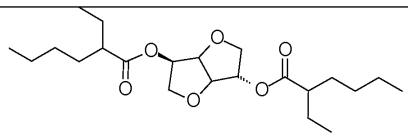
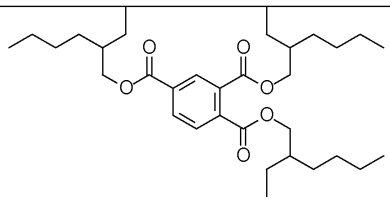
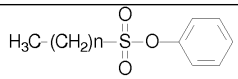
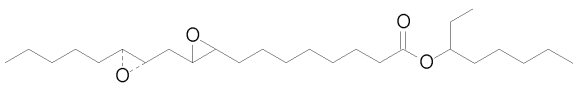
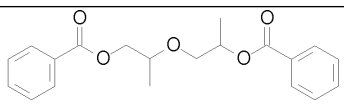
At least two different estrogen receptors (ER) exist, namely, ERα and ERβ (*9*). Besides differences in binding capacities for specific ligands there are differences in tissue distribution of the two receptors. In the mammary gland, uterus, and testes ERα is the predominant receptor (*10*). The ovary contains both receptor subtypes in large amounts, and in the prostate ERβ is the most abundant receptor (*10*). Upon ligand binding homo- and heterodimers of the ERα and ERβ can be formed *in vivo* and *in vitro*, and these dimers can activate the estrogen responsive elements initiating gene expression. It has been suggested that the ERβ might modulate the estrogenic effects of the ERα by forming heterodimers with ERα, thereby reducing the ERα-mediated response, resulting in, for example inhibition of ERα-mediated proliferation of breast cancer cells *in vitro* (*11 - 14*). The inability to obtain a full-length cDNA of the ERβ without mutation (*15*) from mRNA isolated from the human T47D breast cancer and Caco-2 colon cancer cells further supports the idea that ERβ activation may be beneficial and modulate possible adverse ERα mediated estrogenic effects. Given the differential biological responses upon ERα or ERβ activation, it is of importance in the study of endocrine-disrupting compounds to characterize their interaction with both receptors separately. Therefore, the objective of the present study was to characterize the ERα- and ERβ-mediated estrogen response of a wide range of food contaminants and existing and newly developed direct and indirect food additives for their ERα- and ERβ-type estrogenic potential.

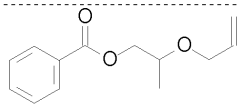
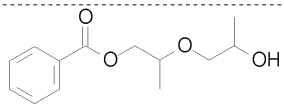
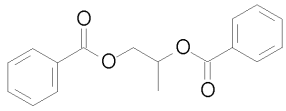
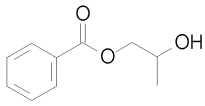
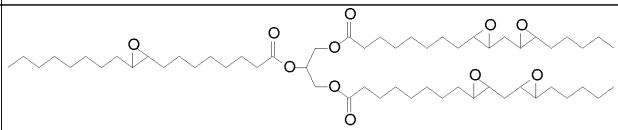
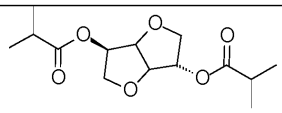
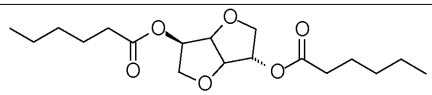
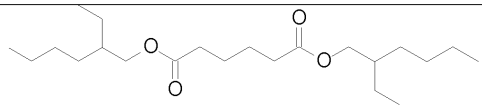
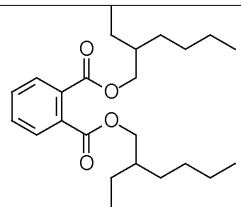
Some endocrine-disrupting compounds are known for their estrogenic activity, such as phthalates (*16 -20*), nonylphenol (NP) (*20 - 24*), and bisphenol A (BPA) (*20, 25 - 29*). Human intake of these and other pseudo-estrogens is mainly via food and originates from natural food chain constituents, containing, for example, pseudo-estrogens, from a contaminated environment (for example, DDT), from food packages (BPA) (*26*), and/or from additives such as antioxidants or plasticizers used in packaging material (*30*).

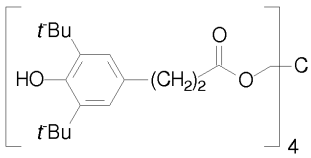
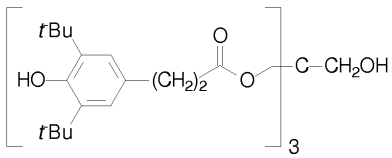
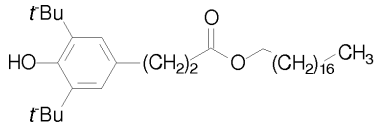
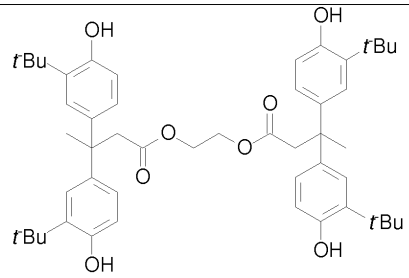
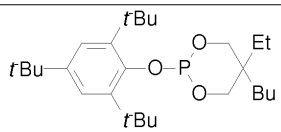
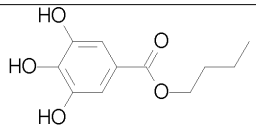
In the present study we determined the ER-type specific estrogenic potency of food-associated compounds by using two different human osteoblastic (U2-OS) reporter gene cell lines. U2-OS cell lines are devoid of endogenous estrogen receptors. The two transfected cell lines stably express either ERα or ERβ in addition to 3xERE-tata-Luc as a reporter gene (*31*).

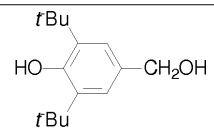
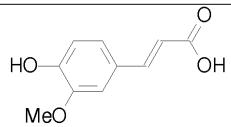
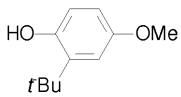
The compounds tested are estradiol (E_2, as a reference), BPA, NP, included in the studies to allow comparison with existing literature data (*23, 32 – 34*), eight existing plasticizers, three newly developed plasticizers based on sorbitol, and nine existing antioxidants. All plasticizers and antioxidants are generally used in food-packaging material and were therefore included in the present study to investigate their estrogenicity, of which little is known so far. Table 1 summarizes and presents all compounds studied.

Table 1. Food-Packaging-Associated compounds tested for their estrogenicity in the ERα and ERβ-reporter gene U2OS assays, including their molecular structure and CAS Registry number. Consecutive numbers for compounds are used to facilitate searching for the compounds in the text.

Compound		Structure	CAS no.
1	17β-estradiol (E_2)		[50-28-2]
2	Bisphenol A (BPA)		[80-05-7]
3	Nonylphenol (NP); 85%, technical mixture	 (4-nonylphenol shown)	[104-40-5]
Plasticizers			
4	Diisoheptyl phthalate (DIHP)		[41451-28-9]
5	Isosorbide di 2-ethylhexanoate (IsDEH)		[94593-67-6]
6	Tris (2-ethylhexyl) trimellitate (>99%) (TEHTM)		[3319-31-1]
7	Phenyl alkylsulfonate	 n= 14-16	[91082-17-6]
8	Octyl epoxy stearate		[128514-16-9]
9. Benzoate mixture (5 compounds)			
9a	Dipropylene glycol dibenzoate (89.4%)		[27138-31-4]

9b	(2-Propenyloxy) propyl benzoate (2.35%)	 D1- Me	[197178-94-2]
9c	Dipropylene glycol monobenzoate (4.98%)	 2(D1- Me)	[32686-95-6]
9d	Propylene glycol dibenzoate (2.29%)		[19224-26-1]
9e	Propylene glycol monobenzoate (0.28%)		[37086-84-3]
10	Epoxidized soy bean oil (ESO)		[8013-07-8]
11	Isosorbide diiso-butyrate (IsDiB)		[125344-74-3]
12	Isosorbide di-*n*-hexanoate (IsDH)		[64896-69-1]
13	Di (2-ethylhexyl) adipate (DEHA)		[103-23-1]
14	Di (2-ethylhexyl) phthalate (DEHP)		[117-81-7]

Antioxidants		Structure	CAS no.
15. Antioxidant mixture consisting of two compounds			
15a	Pentaerythritol tetrakis[3-(3,5-di-tert-butyl-4-hydroxy phenyl)] propionate (>96%)		[6683-19-8]
15b	Pentaerythritol tris [3-(3, 5-di-tert-butyl-4-hydroxy-phenyl)] propionate (<4%)		[84633-54-5]
16	Octadecyl 3-(3,5-di-tert-butyl-4-hydroxy-phenyl)-propionate		[2082-79-3]
17	Ethylene bis[3,3-bis (3-tert-butyl-4-hydroxy phenyl)butyrate]		[32509-66-3]
18	Amines, bis(hydrogenated rape-oil alkyl)methyl, N-oxides	Unknown	[204933-93-7]
19	2,4,6-Tris (tert-butyl) phenyl-2-butyl-ethyl-1,3-propanediol phosphite		[161717-32-4]
20	Propyl gallate		[121-79-9]

21	3,5-Di-tert-butyl-4-hydroxybenzyl alcohol >97%		[88-26-6]
22	4-Hydroxy-3-methoxycinnamic acid (Ferulic acid)		[1135-24-6]
23	Butylated hydroxyl anisole (BHA)		[25013-16-5]

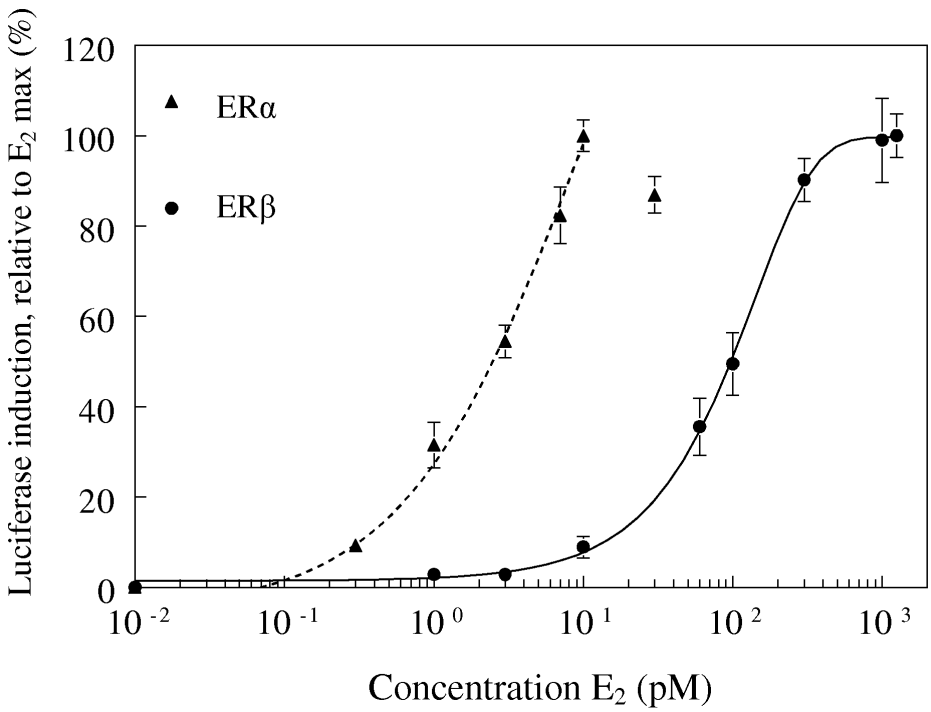


Figure 1
Dose-response curves of U2-OS ERα and U2-OS ERβ cells exposed for 24 h to estradiol (E_2). The responses are expressed as percent relative to the maximum signal induced by E_2 in the same cell line and are corrected for the signal induced by the solvent (DMSO).

Materials en Methods

Chemicals and Reagents

E_2 (>98%), BPA (>99%) and tricine (>99%), were obtained from Sigma (Zwijndrecht, The Netherlands). $NaHCO_3$ (>99.5%), NaOH (>99%), $EDTA{\cdot}2H_2O$ (>99%), $MgSO_4{\cdot}7H_2O$ (>99.5%), 1,4-dithiothreitol (>99%), (L+) ascorbic acid (>99.7%), and diethyl ether (>99.7%) were obtained from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO, >99.9%) was obtained from Acros (Geel, Belgium). Tris(hydroxymethyl)aminomethane (>99.9%) was obtained from Invitrogen (Carlsbad, CA). d-Luciferin (>99.5%) was obtained from Duchefa (Haarlem, The Netherlands). *trans*-1,2-Diaminocyclohexane-*N,N,N',N'*-tetraacetic acid monohydrate (CDTA, >99%) and NP (~85%; technical mixture) were obtained from Fluka (Buchs, Germany). $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$ was obtained from Aldrich (Milwaukee, WI). Di(2-ethylhexyl) phthalate (DEHP) was obtained from TCI (Tokyo, Japan). ATP was obtained from Roche (Mannheim, Germany). All plasticizers and antioxidants (Table 1) are of technical grade. To enable the reader to search for the compounds in Table 1, all compounds are numbered in sequence. Sorbitol-based plasticizers were produced by Agrotechnology and Food Innovations, Wageningen UR, in the project sugar polyol based plasticizers, aiming at the development of efficient, undisputed, renewable esters as plasticizers and solvents. CAS Registry numbers are given in Table 1. All chemicals were dissolved and diluted in DMSO. Food-packaging-associated compounds were tested at least two times in five concentrations ranging from 0 to $5x10^{-5}$ M. As *in vivo* exposure to pseudo-estrogens always occurs in combination with the presence of endogenous hormones the compounds were also tested with E_2 present at its EC_{50} value in both cell lines. To this end the cells were incubated with the tested compounds at doses ranging from 0-$5x10^{-5}$ M in the presence of E_2 at its EC_{50} value for agonist action.

Cell Culture

U2-OS ERα and ERβ cells (*31*) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixture F12 (31331-028, Gibco), buffered with 1260 mg/L $NaHCO_3$, supplemented with 7.5% fetal calf serum (FCS, Australian origin, 10099, Invitrogen) and 0.5% nonessential amino acids (minimal essential medium 100x, 11140-035, Gibco). ERα medium was supplemented with geneticin (200 µg/mL, Invitrogen) and hygromycin (50 µg/mL, Duchefa) as selection markers. ERβ medium was supplemented with geneticin (200 µg/mL) only. Cells were cultured at 37°C at 7.5% CO_2 in a humidified atmosphere.

Luciferase Assay

U2-OS cells were washed twice with phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}; Gibco), trypsinized (Gibco) and diluted in a 1:1 mixture of DMEM and Ham's F12 medium without phenol red (21041-025, Gibco), buffered with 1260 mg/L $NaHCO_3$, supplemented with 5% dextran-coated charcoal (DCC)-stripped FCS (*36*) and 0.5% nonessential amino acids. ERα cells were seeded at a density of 10000 cells/well and ERβ cells were seeded at a density of 7500 cells/well in 96-well Nunclon plates (167008, NUNC). After 24 h,

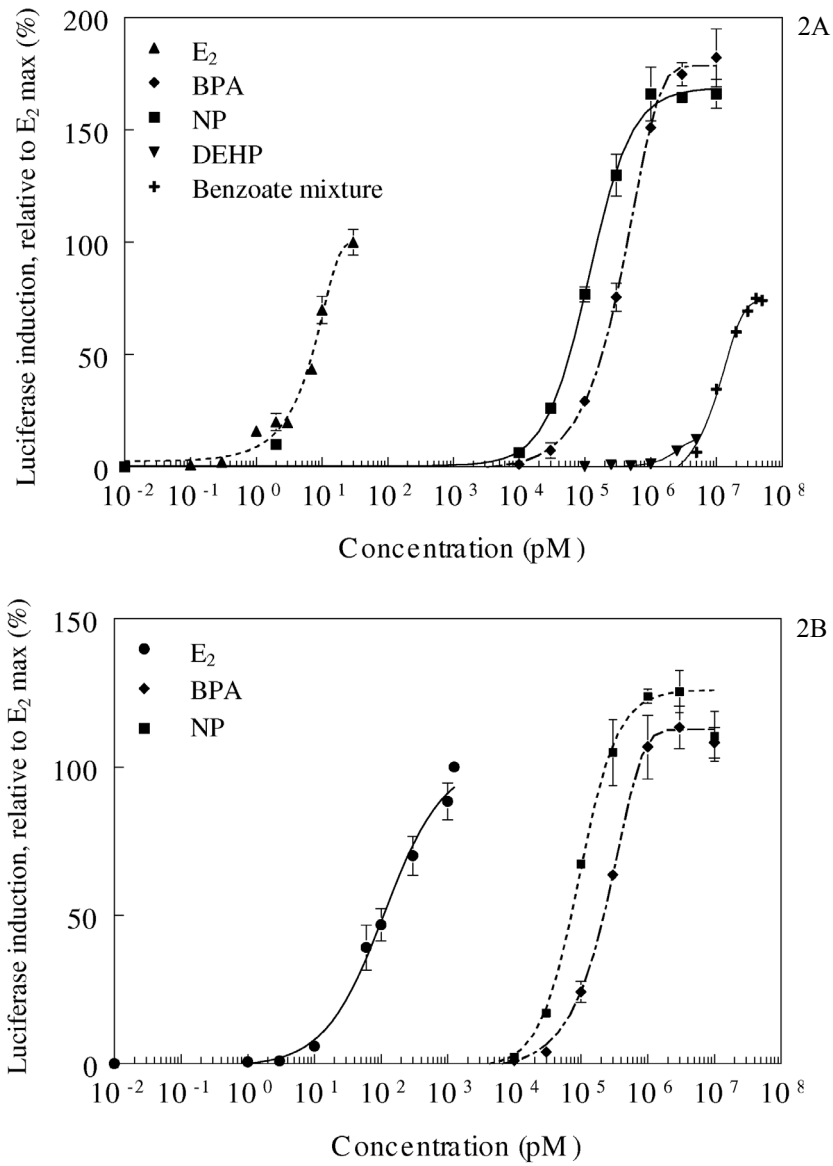


Figure 2
Dose-dependent induction of luciferase activity (2A) in the ERα cell line and (2B) in the ERβ cell line by bisphenol A (BPA), nonylphenol (NP), di(2-ethylhexyl)phthalate (DEHP), and a benzoate mixture. DEHP and the benzoate mixture did not induce luciferase activity in the ERβ cell line. The responses are expressed as percent relative to the maximum signal induced by E_2 in the same cell line and are corrected for the signal induced by the solvent (DMSO).

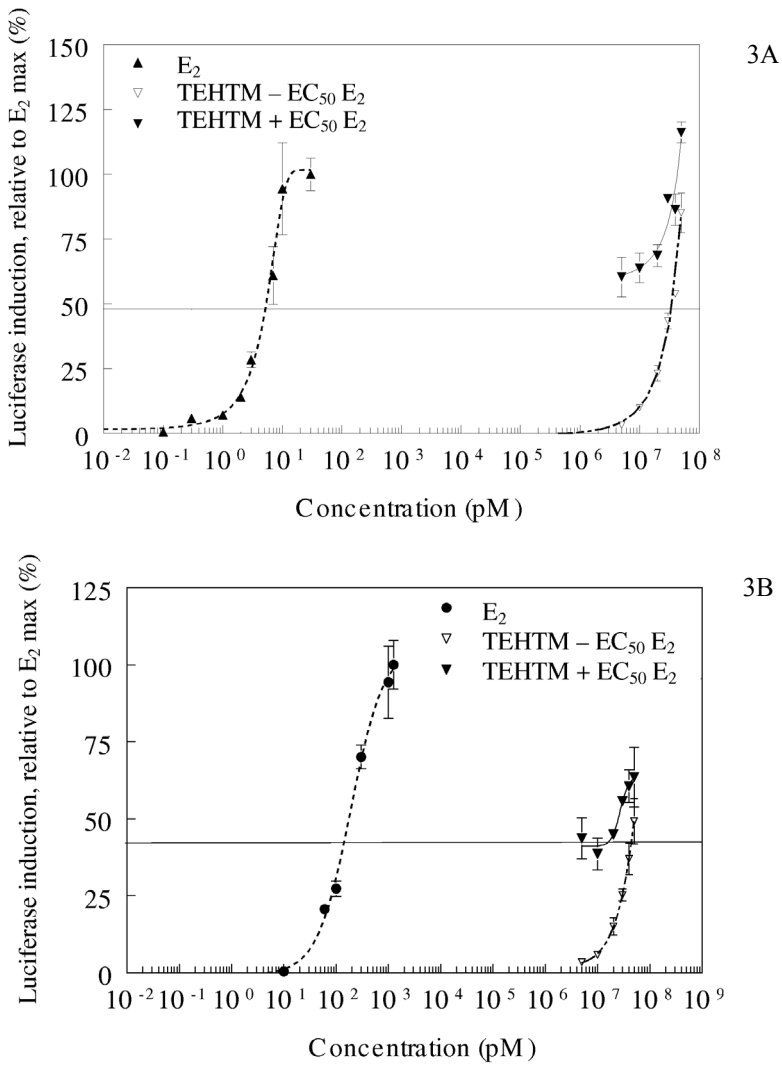


Figure 3
Dose-response curve of (3A) ERα cells exposed for 24 h to estradiol (E_2), tri-2-ethylhexyl-trimellitate (TETHM) alone, and TEHTM + 5 pM E_2 (straight line indicates the EC_{50} level of E_2) and (3B) ERβ cells exposed for 24 h to E_2, TETHM alone, and TEHTM + 100 pM E_2 (straight line indicates the EC_{50} level of E_2). The responses are expressed as percent relative to the maximum signal induced by E_2 in the same cell line and are corrected for the signal induced by the solvent (DMSO).

assay medium was renewed, and 24 h later, assay medium was removed and replaced by 100 µL of exposure medium, with test compounds dissolved in DMSO concentrations of 0.1%. For combination experiments DMSO concentrations were 0.2%. All experiments with antioxidants used 700 µM ascorbic acid in the exposure medium to prevent autoxidation. Compounds were tested in triplicate; outer rows were filled with 200 µL of sterile PBS to prevent effects of evaporation on the outer side of the plates. After 24 h of exposure, medium was removed and cells were washed with 0.5·PBS. Cells were lysed with 30 µL of hypotonic low-salt buffer (10 mM Tris, 2 mM DTT, and 2 mM CDTA; pH 7.8). Plates were put on ice for 15 min to allow swelling of the cells and subsequently frozen at –80°C for at least 30 min to lyse the cells. Plates were thawed on ice and shaken for 2 min at room temperature. Luciferase activity was measured at room temperature in a luminometer (Labsystems, Luminoskan RS). First, background light emission was measured for 2s, then 100 µL of "flashmix" [20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM $EDTA \cdot 2H_2O$, 2 mM DTT, 0.47 mM d-luciferin, and 5 mM ATP; pH 7.8] was automatically injected, and light emission was immediately measured during 2s and extinguished with 50 µL of 0.2 M NaOH to stop the reaction.

Table 2. EC_{50} and EEF_{50} values of nonylphenol (NP) and bisphenol A (BPA) compared to estradiol (E_2), as determined in the U2-OS ERα and U2-OS ERβ cell lines.

Compound	Assay	EC_{50} (nM)	EEF_{50}	EEF_{50}α/ EEF_{50}β	Max. effect as % relative to E_2-max
E_2	ERα	0.006±0.002	1	1	100
	ERβ	0.124±0.03	1		100
NP	ERα	124±63	$5.3 \cdot 10^{-5}$	0.054	114±26
	ERβ	149±57	$8.3 \cdot 10^{-4}$		122±10
BPA	ERα	216±35	$3.1 \cdot 10^{-5}$	0.094	170±17
	ERβ	234±110	$5.3 \cdot 10^{-4}$		116±11

Data Analysis

Relative light units (RLUs) in every well were corrected for the corresponding background signal, measured before luciferin addition. To be able to compare all data, the response of the solvent control (0.1% DMSO for single and 0.2% for combination experiments) was set at 0%. The maximum induction of luciferase obtained at 10 pM E_2 for ERα cells and at 1250 pM E_2 for ERβ cells was set at 100%. In every experiment an E_2 calibration curve was included using 0, 0.3, 1, 2, and 10 pM E_2 for ERα and 0, 10, 60, 100, and 1250 pM E_2 for ERβ. Dose-response curves were fitted using Slidewrite 6.10 for Windows.

Estradiol equivalency factors (EEF_{10} = $EC_{10\ estradiol}/EC_{10\ compound}$) were calculated for the responses in ERα and ERβ cell lines. EC_{10} values were interpolated from the E_2 calibration curves; the EC_{10} values were used instead of EC_{50} values because for less potent compounds factors such as insolubility hamper measurements at higher concentrations. For the more potent compounds, such as BPA and NP, also EEF_{50} values were calculated as $EC_{50\ estradiol}/EC_{50\ compound}$ (Table 2).

Table 3. Lowest effect concentration (LEC, concentration that equals the effects of DMSO + 3 x SD), Estradiol Equivalence Factors (EEF_{10}), and the maximum effect (%) of all estrogenic compounds tested positive within the present study compared to E_2.

	ERα			ERβ			
Compounds	**LEC (μM)**	**EEF_{10}**	**Max effect %**	**LEC (μM)**	**EEF_{10}**	**Max effect %**	**$EEF_{10}α$/ $EEF_{10}β$**
E_2	$0.3 \cdot 10^{-6}$	1	100	$6.6 \cdot 10^{-6}$	1	100	1
NP	0.25	$4.6 \cdot 10^{-5}$	104	0.01	$5.2 \cdot 10^{-4}$	121	0.09
BPA	0.18	$2.5 \cdot 10^{-5}$	193	0.02	$2.3 \cdot 10^{-4}$	117	0.11
Plasticizers							
DIHP	2.3	$3.1 \cdot 10^{-7}$	33	a)	a)	a)	
DEHP	0.97	$2.2 \cdot 10^{-7}$	14	a)	a)	a)	
DEHA	25.4	$4.9 \cdot 10^{-8}$	16	1.7	b)	3.1	
TEHTM	8.1	$1.2 \cdot 10^{-7}$	113	4.9	$1.6 \cdot 10^{-6}$	76	0.075
Octyl-epoxystearate	7.9	$1.3 \cdot 10^{-8}$	26	a)	a)	a)	
Benzoate mixture	3.7	$2 \cdot 10^{-7}$	72	a)	a)	a)	
Antioxidants							
Propyl gallate	2.1	$6.5 \cdot 10^{-7}$	109	2.5	$3.9 \cdot 10^{-6}$	60	0.17
Propanediol phosphite	31.7	$1.2 \cdot 10^{-8}$	7.4	a)	a)	a)	
BHA	5.9	$5.2 \cdot 10^{-8}$	18.3	8.4	$7.7 \cdot 10^{-7}$	15.6	0.07

a) Activity below limit of quantification (DMSO+3*STDEV)
b) Activity too low to calculate an EEF_{10}

Responses were taken into account only when they were above the limit of quantification (LOQ), defined as the DMSO response plus 3 times its standard deviation (SD). The limit of detection (LOD) was defined as the DMSO response plus 2 times its SD. The LOQ interpolated in the E_2 curve gave the lowest effect concentration (LEC).

Results

Estradiol (1, E_2) Calibration Curve:
Exposure of the ERα and ERβ cell lines to increasing concentrations of E_2 (1) resulted in sigmoidal dose-response curves (Figure 1). The LEC for ERα cells was 0.3 pM (± 0.3), and the LEC for ERβ cells was 6.6 pM (± 3.9). EC_{50} values differ almost 20-fold being 6.6 (± 1.6) pM for ERα cells and 124.2 (±29.6) pM for ERβ cells. The maximum RLU value in the ERα cell line was 5 times higher than the maximum RLU value in the ERβ cell line. When expressed as induction relative to DMSO, however, the maximum induction of the ERβ cell line was 12 times higher than that in the ERα cell line, because of the relatively high background signal in the ERα. To be able to compare dose response curves, results are presented on scales from 0 to 100% induction by E_2 (Figure 1). From Figure 1 it can be derived that at 30 pM E_2 luciferase induction in the ERα cells declines, and therefore this data point was not included in defining the dose response curve.

Table 4. Overview of EC_{50} and EEF_{50} values of nonylphenol (NP) and bisphenol A (BPA) compared to estradiol (E_2), as reported in the literature for reporter gene and proliferation mammalian cell models.

Compound	Assay	EC_{50} (nM)	EEF	%Max. effect rel. to E_2	Reference
E_2	ERα	0.02	1		*35*
	ERβ	0.06	1		*31*
	ER-CALUX	0.006	1	100	*23*
	E-screen	0.005	1	a	*32*
	E-screen	0.005	1	a	*33*
	MVLN-cells	0.005	1	a	*33*
	MVLN-cells	0.015	1	a	*34*
	HGELN cells	0.04	1	a	*33*
NP	ER-CALUX	260	$2.3 \cdot 10^{-5}$	135	*23*
	E-screen	400	$1.3 \cdot 10^{-5}$	a	*33*
	MVLN-cells	400	$1.3 \cdot 10^{-5}$	a	*33*
	MVLN-cells	463	$3.2 \cdot 10^{-5}$	a	*34*
	HGELN cells	500	$8 \cdot 10^{-5}$	a	*33*
BPA	ER-CALUX	770	$7.7 \cdot 10^{-6}$	153	*23*
	E-screen	410	$1.1 \cdot 10^{-5}$	a	*32*
	E-screen	200	$2.5 \cdot 10^{-5}$	a	*33*
	MVLN-cells	200	$2.5 \cdot 10^{-5}$	a	*33*
	HGELN cells	210	$1.9 \cdot 10^{-4}$	a	*33*

a: Not given in literature.

Food-Packaging-Associated Compounds:

Figure 2 presents the results from a representative experiment showing the luciferase induction in ERα and ERβ cells upon exposure to different food-packaging-associated compounds. The maximum luciferase induction by BPA (2) and NP (3) is higher than the maximum luciferase induction by E_2, especially in the ERα cells (Figure 2). Table 2 presents the EC_{50} and EEF_{50} values obtained for E_2, NP, and BPA from a series of four similar independent experiments. To compare results obtained in the ERα and ERβ cell line Table 2 also presents the ratio $EEF_{10}\alpha/EEF_{10}\beta$, which, by definition, equals 1 for E_2. If the ratio $EEF_{10}\alpha/EEF_{10}\beta$ of a compound is <1, this compound is relatively more estrogenic in the ERβ cell line, showing, compared to E_2, a relatively higher EEF_{10}, in the ERβ than in the ERα cell line. The $EEF_{10}\alpha/EEF_{10}\beta$ ratios of BPA and NP are ~0.1, meaning they are relatively more estrogenic as compared to E_2 in the ERβ cell line than in the ERα cell line.

The results of tested compounds that show activity in the ERα and ERβ cell lines are summarized in Table 3. All sorbitol-based plasticizers [isosorbide-di-2-ethylhexanoate (5), isosorbide diiso-butyrate (11), isosorbide-di-*n*-hexanoate (12)], the phenyl alkylsulfonate (7) and epoxidized soybean oil (ESO, 10) appeared to be nonestrogenic in both cell types.

Nonestrogenic antioxidants were the antioxidant mixture [96% pentaerythritol tetrakris[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)]propionate (15a) and 4% pentaerythritol tris[3-(3,5-di-*tert*-butyl-4-hydroxy-phenyl)]propionate (15b)], octadecyl 3-(3,5-di-tert-butyl-4-hydroxy-phenyl)propionate (16), ethylene bis[3,3-bis(3-*tert*-butyl-4-hydroxyphenyl)-butyrate] (17), bis(hydrogenated rape-oil alkyl)-methyl *N*-oxides (18), 3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol (21), and *trans*-4-hydroxy-3-methoxycinnamic acid (ferulic acid) (22).
Five plasticizers appeared to be estrogenic in the ERα cell line and not in the ERβ cell line; these include di-isoheptyl phthalate (DIHP [4]), di (2-ethylhexyl) phthalate (DEHP, 14), di(2-ethylhexyl)adipate (DEHA, 13), octylepoxy stearate (8) and the benzoate mixture [89.4% dipropyleneglycol dibenzoate (9a), 2.35% (2-propenyloxy) propyl benzoate (9b), 4.98% propyleneglycol dibenzoate (9c), 2.29% propyleneglycol benzoate (9d), and 0.28% propyleneglycol monobenzoate (9e)]. Tris(2-ethylhexyl) trimellitate (TEHTM, 6) was estrogenic in both cell lines with an $EEF_{10}\alpha/EEF_{10}\beta$ ratio of 0.075. The estrogenic potencies of positive compounds as compared to E_2 can all be found in Table 3.
The antioxidants propyl gallate (20) and butylated hydroxy anisole (BHA, 23) were estrogenic in the ERα cells as well as in the ERβ cells with $EEF_{10}\alpha/EEF_{10}\beta$ ratios of 0.17 and 0.07, respectively. The antioxidant 2,4,6-tri-*tert*-butylphenyl-2-butyl-ethyl-1,3-propanediolphosphite (propanediolphosphite, 19), was estrogenic only in the ERα cells (Table 3).

Effects of Combined Exposures:
The compounds shown to be active in the ERα and ERβ cells were tested in the presence of E_2 at its EC_{50} level to test whether the responses of these combined exposures are additive. Panels A and B of Figure 3 show the results obtained for TEHTM (6) in the ERα and ERβ cell lines, respectively. Additive effects were observed and similar data were obtained for the other compounds shown to be active in the ERα or ERβ cell line when tested alone.

Discussion

The present study investigated the ER-type specific estrogenic potency of some foodborne compounds using two different U2-OS reporter gene cell lines stably expressing either ERα or ERβ in addition to 3xERE-tata-Luc (*31*). The results obtained reveal differences in estrogen activity toward both receptor types of different estrogenic compounds as measured with the U2-OS ERα and U2-OS ERβ cell lines. The EC_{50} of E_2 (1) in the ERα cell line appeared to be 20 times lower than the EC_{50} of E_2 in the ERβ cell line. E_2 tested in an ERα and ERβ-transfected human embryonic kidney (HEK) cell line showed a comparable difference in activation (*37*). As can be seen in Table 4, presenting an overview of EC_{50} values for E_2, BPA (2) and NP (3) reported in the literature based on other mammalian reporter- and estrogen-dependent cell proliferation models, the EC_{50} values reported in these literature studies are more in line with the EC_{50} obtained in the present study in the ERα cells than with the EC_{50} for the ERβ cells (Table 2). This suggests that the T47D reporter cell line (ER-CALUX) (*23*), E-screen (*32, 33*), and MVLN cell assays (*33, 34*) detect primarily ERα-type responses. However, as these cell lines are not specifically free of other endogenous steroid receptors, interactions between the ER, the androgen receptor, and even the progesterone

receptor cannot be excluded. This further supports the importance of detecting characteristics for ERβ response in an independent cell model such as the U2-OS ERβ model used in the present study. This ERα-type response in the ER-CALUX, E-screen, and MVLN cell assays could be due to the fact that all of these cell systems mainly express endogenous ERα and estrogenicity is dependent on the most abundant receptor (*14, 32*). The luciferase induction by BPA of more than the maximum induction by E_2 (set at 100%) in the U2-OS ERα cells is in accordance with the induction previously shown in the T47D reporter cell line (ER-CALUX) (*23*) (Table 4). Legler *et al.* suggested that this "superagonism", that is, a maximal response being higher than that induced by E_2, could be due to stimulated receptor and/or cofactor renewal (*23*). As the U2-OS cell line is devoid of endogenous receptors, it can be excluded that there is cross-reactivity of different receptors. Another explanation could be that BPA modulates the activity of several kinases involved in ERE activation. To gain more insight into the mechanism underlying this overstimulation, molecular studies are needed, which are, however, beyond the scope of the present study.

Recently Gustafsson and co-workers demonstrated, using the breast cancer cell line T47D with tetracycline-dependent inducible ERβ expression in addition to the ERα, that ERβ expression inhibits proliferation induced by estradiol at 10 nM, and by the drugs 4-hydroxytamoxifen and raloxifene at 1 μM (*14*). This suggests that ERβ can influence the cell proliferation induced by ERα. In the present study, BPA and NP are 10 times more estrogenic in the ERβ cell line, compared to E_2, than in the ERα cell line. This is reflected by an $EEF_{10}\alpha/EEF_{10}\beta$ ratio of 0.1, which indicates that in theory BPA and NP might to some extent modulate the negative estrogenic effects via the ERα, because of their relatively high-affinity binding to the ERβ.

For BPA a specific migration limit of 0.05 mg/kg of food and a tolerable daily intake (TDI) of 0.01 mg/kg of body weight (bw)/day have been established. This TDI is above the average consumer intake, which is estimated by the SCF to range from 0.00048 mg/kg of bw/day for adults to 0.0016 mg/kg of bw/day for infants in a realistic estimate of exposure via food (*38*). Average intake of nonylphenol was estimated by Geunther *et al.* to be 7.5 μg/person/day, which would be equal to 125 ng/kg of bw/day for a 60 kg person (*39*).

Various plasticizers, including DIHP (4), DEHP (14), DEHA (13), TEHTM (6), octyl-epoxystearate (8) and the benzoate mixture (9) showed estrogenic activity *in vitro*, activating the ERα with EEF_{10} values between 10^{-8} and 10^{-7}. Of all of these compounds, only TEHTM was estrogenic in the ERβ cells, revealing an $EEF_{10}\alpha/EEF_{10}\beta$ comparable to those of NP and BPA. For DEHP human exposure is estimated to range from 30 μg/kg of bw/day in the regular population to 457 μg/kg of bw/day in hemodialysis patients (*40*). For DEHP the TDI of the general population is set at 0.05 mg/kg of bw/day (*41*). The specific migration limit of DEHA is 18 mg/kg of food and the TDI is 0.3 mg/kg of bw/day. The median intake in The Netherlands and other western European countries is ~1 mg/day; this means 16.6 μg/kg of bw/day for a 60 kg person (*42*).

The compounds showing estrogenic activity in our study have been tested in several other models for their estrogenic activity, showing variable results. DIHP was not found to be estrogenic *in vitro* in an estrogen receptor competitive ligand binding and mammalian- and yeast-based gene expression assays (*43*). On the other hand, a small estrogenic *in vivo* effect of DIHP on uterine wet weight in ovariectomized Sprague-Dawley rats was found (*43*). Takeuchi *et al.* (*44*) reported DIHP and DEHP to be active in an ERα-transfected CHO

cell line with comparable EEF_{20} values of, respectively $6.3 \cdot 10^{-6}$ and $5.5 \cdot 10^{-6}$. The difference with our EEF_{10} values can be due to the different cell types used or to differences in the interpolation of EC_{10} or EC_{20} values. DEHP was not estrogenic in the T47D ER-CALUX cell line (*45*); this discrepancy with our results in the ERα cells could be due to a relative overexpression of the ERα receptor in our U2-OS transfected cell line compared to the T47D cell line with endogenous ERα expression used in the ER-CALUX.

With respect to the safety assessments of the various plasticizers tested, the following findings of the present study are of interest. DEHA appeared to be less estrogenic ($EEF_{10}\alpha$ of $4.9 \cdot 10^{-8}$) than DEHP ($EEF_{10}\alpha$ of $2.2 \cdot 10^{-7}$) (Table 3); therefore, DEHA would be preferable over DEHP to use in food packaging with regard to the estrogenic effect only. However, given the current concern about the migration of adipates (*46, 47*), substances such as DEHA cannot be considered as viable alternatives to DEHP in food-contact applications, despite the result with our estrogenicity assay. Of course, other mechanisms for endocrine disruption, such as (anti)androgenicity, have to be taken into account as well.

Octyl-epoxystearate (8) is used as a secondary plasticizer in PVC, as is epoxidized soybean oil (ESO, 10). As ESO is not estrogenic at all and octyl-epoxystearate very slightly estrogenic in our ERα cells, ESO seems to be a better choice to use as a plasticizer (Table 3). Our results, obtained with all plasticizers used in food packaging that are currently in use, reveal that several of these plasticizers are slightly estrogenic. Three newly developed plasticizers based on sorbitol, isosorbide di 2-ethylhexanoate (5), isosorbide-diiso-butyrate (11), and isosorbide-di-*n*-hexanoate (12) activated neither the ERα nor the ERβ. Therefore, as far as estrogenicity is concerned, these plasticizers may be good replacements of existing plasticizers. However, additional data on these newly developed plasticizers regarding persistence, migration, toxicity and accumulation in food products are necessary to be able to fully judge the added value of replacement.

Of the antioxidants, propanediolphosphite (19) was slightly estrogenic with an EEF_{10} of $1.2 \cdot 10^{-8}$ in the ERα cell line. Of all antioxidants tested, propyl gallate (20) and BHA (23) were the only ones revealed to be estrogenic in both the ERα and ERβ cells. As can be noted again, their potencies in the ERα were lower compared to the ERβ-mediated activity, especially for BHA (Table 3). For these compounds, BHA exposure is estimated to be 0.11 mg/person/day (*48*), and the ADI ranges from 0.3 to 0.5 mg/kg of bw/day (*30*). Propyl gallate's ADI is 1.4 mg/kg of bw/day (*30*).

The response of the test compounds in the ERα and ERβ cell lines in the presence of the EC_{50} value (5 and 100 pM for the ERα and ERβ cell lines, respectively) revealed that all combination effects were additive. This indicates that estrogenic effects of compounds *in vitro* are additive, a finding which should be studied *in vivo* as well. The concentrations of E_2 used for these combined exposures (5 and 100 pM) are commonly found in female blood, and they are even low compared to E_2 levels during pregnancy (*49*).

All together the results of the present study reveal that plasticizers have estrogenic activity in the ERα U2-OS reporter cell line, although at ~100 times higher concentrations than those of the known pseudo-estrogens BPA and NP. However, because additivity seems to occur and all of these compounds are regularly used in food packages, together they may contribute significantly to the estrogenic effects of the total pool of compounds with estrogenic activity originating from the food chain. Most compounds are estrogenic in the ERα cell line, indicating

that the effects predominantly will occur in tissues expressing ERα, such as mammary gland, uterus, and testes (*10*). When compared to the effects of E_2 (1), for NP (3), BPA (2), TEHTM (6) and propyl gallate (20), the EEF_{10} is relatively higher in the ERβ than in the ERα, resulting in an $EEF_{10}\alpha/EEF_{10}\beta$ ratio lower than 1. This indicates that these compounds might give rise to relatively larger formation of ERα/ERβ heterodimers than upon exposure to E_2, thereby possibly diminishing the negative effects of the ERα as seen *in vitro* (*12, 13*). The plasticizers, DIHP (4), DEHP (14), DEHA (13), octyl epoxystearate (8), and the benzoate mixture (9) and the anti-oxidant propanediolphosphite (19) are all exclusively estrogenic via the ERα and might therefore only have adverse endocrine-disrupting effects on human health.
Finally, comparison of the EC_{50} values obtained in the ERα and ERβ U2-OS cells to the EC_{50} values obtained in other models testing estrogenic activity indicates that the EC_{50} values in most of these other cell systems are more in line with the EC_{50} obtained in the ERα cells than with the EC_{50} for the ERβ cells. This observation supports the importance of detecting characteristics for ERα and ERβ response separately in independent models such as the U2-OS ERα and ERβ cells, as done in the present study. Food-packaging-associated compounds are estrogenic at concentrations 100 times higher than the known compounds NP and BPA. The results obtained in this study are important in determining the different reactions of the same compounds on two different types of ER. Both ERs are present in different tissues and able to modulate each other's activity. For most compounds daily intake estimates are in the range from 0 to 0.457 µg/kg of bw/day, and therefore the concentrations tested *in vitro* were high compared to the average human intake; however, they were important in determining the intrinsic potential of these compounds to act as estrogenic agonists. To be able to translate the *in vitro* findings to the *in vivo* situation, currently studies are being performed with ER reporter gene mice to establish the estrogenicity of some of the estrogenic food-packaging-associated compounds *in vivo*.

Abbreviations:

U2-OS, human osteoblastic cell line; ERα/β, estrogen receptor alpha/beta; ERE, estrogen responsive elements; Luc, luciferase gene; EC_{50}, concentration at which 50% of the effect is reached; pM, picomolar; DDT, dichlorodiphenyltrichloroethylene; RLU, relative light units; DCC, dextran-coated charcoal

Keywords:

Antioxidant, endocrine disrupter, ERα, ERβ, estrogen, food packaging, plasticizer, pseudo-estrogen.

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Chapter 3:

Food associated estrogenic compounds induce estrogen receptor mediated luciferase gene expression in transgenic male mice.

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3

Abstract

The present paper aims at clarifying to what extent seven food associated compounds, shown before to be estrogenic *in vitro,* can induce estrogenic effects in male mice with an estrogen receptor (ER)-mediated luciferase (luc) reporter gene system. The luc induction was determined in different tissues eight hours after dosing the ER-luc male mice intraperitoneally (IP) or fourteen hours after oral dosing. Estradiol-propionate (EP) was used as a positive control at 0.3 and 1 mg/kg bodyweight (bw), DMSO as solvent control. The food associated estrogenic compounds tested at non-toxic doses were: bisphenol A (BPA) and nonylphenol (NP) (both at 10 and 50 mg/kg bw), dichlorodiphenyldichloroethylene (*p,p'*-DDE; at 5 and 25 mg/kg bw), quercetin (at 1.66 and 16.6 mg/kg bw), di-isoheptyl phthalate (DIHP), di-(2-ethylhexyl) phthalate (DEHP) and di-(2-ethylhexyl) adipate (DEHA) all at 30 and 100 mg/kg bw. In general IP dosing resulted in higher luc inductions than oral dosing. EP induced luc activity in the liver in a statistically significant dose-related way with the highest induction of all compounds tested which was 20,000 times higher than the induction by the DMSO-control. NP, DDE, DEHA and DIHP did not induce luc activity in any of the tissues tested. BPA induced luc in the liver up to 420 times via both exposure routes. BPA, DEHP and quercetin induced luc activity in the liver after oral exposure. BPA (50 mg/kg bw IP) also induced luc activity in the testis, kidneys and tibia. The current study reveals that biomarker-responses in ER-luc male mice occur after a single oral exposure to food associated estrogenic model compounds at exposure levels 10-10^4 times higher than the established TDI's for some of these compounds. Given the facts that i) the present study did not include chronic exposure and that ii) simultaneous exposure to multiple estrogenic compounds may be a realistic exposure scenario, it remains to be seen whether this margin is sufficiently high.

Introduction

Estrogenic compounds are studied intensively, as there is concern about their possible endocrine disrupting effects (*1*). For the quantification of the estrogenicity of pseudo-estrogens several *in vitro* assays have been developed. Very specific and sensitive *in vitro* assays are estrogen receptor (ER)-mediated reporter gene assays using genetically modified cell lines (*2, 3*). In general pseudo-estrogens are 10^4-10^8 less estrogenic than the natural female hormone estradiol (E_2), (*2, 3*). The *in vivo* relative estrogenicity (compared to E_2), however, can be much higher or lower as has been shown respectively for example for *o,p'*-DDT, ethynylestradiol, and nonylphenol (NP) in transgenic zebrafish (*4*).
For human risk assessment it is necessary to know whether food associated compounds which have been shown to be estrogenic *in vitro* (*3*), are also able to induce estrogenic effects *in vivo*. For instance, the *in vitro* estrogenicity for phthalates appeared not to be predictive for their *in vivo* estrogenicity (*5*). The current research project aims at clarifying to what extent food associated compounds, shown to be estrogenic in ERα and ERβ-specific *in vitro* estrogen receptor (ER)-mediated reporter gene cell lines, can induce estrogenic effects *in vivo* in male ER-luciferase (ER-luc) reporter gene mice. The ER-luc mice contain a 3xERE-tata-Luc insulated reporter gene construct, which reveals the activation of the endogenous estrogen receptor, as has been described (*6, 7*). In these studies dosing was intraperitoneally (IP) which has the advantage that the compound has a high chance of reaching the circulation and tissues (*6, 7*). This may not be the case upon oral exposure. Low intestinal uptake and first pass metabolism could strongly reduce the systemic concentration, resulting in differences when comparing the effects of orally dosed compared to IP dosed compounds. Therefore in this study the mice were exposed both IP as well as orally and the ER-mediated luc induction was determined in several tissues of the animals. It has been shown that the *in vivo* luc response is very fast, as only 8 hours were needed after IP exposure to estradiol-propionate (EP) and diethylstilbestrol (DES) for maximum luc induction in the liver (*6*). In this study we determine luc activity 8 hours after IP-exposure and 14 hours after oral exposure to allow time for uptake and response. Exposure periods are expected to be short enough to prevent feedback mechanisms and indirect effects, such as down-regulation of receptors and relevant genes, to become too important. Male mice were chosen because of their relative low background levels of natural estrogens. Seven, *in vitro* estrogenic, food associated compounds were tested for their *in vivo* estrogenicity, 17β-estradiol 3,17-dipropionate was used as a positive control. The main aim of the current experiment was to determine whether the *in vitro* estrogenic compounds would also be estrogenic *in vivo* via either the more relevant oral exposure route and the worst-case IP exposure which is commonly used for experiments in ER-luc reporter gene mice. The lowest dose inducing an estrogenic response *in vivo* in the present study is compared to the tolerable daily intake for estrogenic compounds in food as far as these were available for the model compounds of the present study.

Animals, Materials and Methods

Chemicals and reagents
The estrogenic compounds tested were 17β-estradiol 3,17-dipropionate (EP, >98%, CAS:

113-38-2), bisphenol A (BPA, >99%, CAS: 80-05-7), *p,p'*-DDE (>99%, CAS: 72-55-9), obtained from Sigma (Zwijndrecht, The Netherlands), quercetin dihydrate (99%, CAS: 6151-25-3) from Acros Organics (Geel, Belgium), nonylphenol (NP, ~85% technical mixture, CAS: 104-40-5) from Fluka (Buchs, Germany), di-(2-ethylhexyl) phthalate (DEHP) (CAS: 117-81-7) from TCI (Tokyo, Japan). Diisoheptyl phthatale (DIHP, CAS: 41451-28-9) and di-(2-ethylhexyl) adipate (DEHA, CAS: 103-23-1) were technical grade and obtained from Agrotechnology and Food Innovations (Wageningen, The Netherlands).
Further chemicals used were tricine (>99%), glycylglycine, ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetra-acetic acid (EGTA), Triton X-100, bovine serum albumin (BSA, >97%) and corn-oil all from Sigma (Zwijndrecht, The Netherlands). Sodium hydroxide (NaOH, >99%), EDTA·$2H_2O$ (>99%), $MgSO_4 \cdot 7H_2O$ (>99,5%) and 1,4-dithiothreitol (>99%) were obtained from Merck (Darmstadt, Germany). D-luciferin (>99,5%) was obtained from Duchefa (Haarlem, The Netherlands). $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$ was obtained from Aldrich (Milwaukee, WI, USA). ATP was obtained from Roche (Mannheim, Germany).
All chemicals were dissolved and diluted in dimethyl sulfoxide (DMSO > 99,9%) purchased from Acros Organics (Geel, Belgium).

Animal experiment
The male ER-luc reporter gene mice originated from the stable reporter line INS7 (*6*) and were randomly bred within our colony. The mice were fed a phyto-estrogen free diet throughout their life (AM-II diet, Hope Farms, The Netherlands). Water was provided in glass bottles. Water and food were provided *ad libitum*. The animal experiment was approved by the Animal Ethics Committee of Wageningen University. Six animals were used per group for IP exposure. Eight animals were used per group for oral exposure. Exceptions in number of animals were for oral exposure only: the DMSO control group consisted of 9 mice, the groups exposed to 5 or 25 mg/kg bw *p,p'*-DDE consisted of 4 mice each, and the groups exposed to 100 mg/kg bw DEHA or 1.66 mg/kg bw quercetin consisted of 7 mice. The weight of the animals ranged from 21.8 to 57.9 g for the mice in the oral exposure groups and from 24.2 to 49.4 g for the mice in the IP exposed groups.
For intraperitoneal (IP) exposure DMSO stock solutions of the compounds were diluted to 5% in corn-oil and 6.66 µl/gram bodyweight (bw) of this solution was injected IP once. Eight hours after IP dosing, comparable to Lemmen *et al.* (*6, 7*), male mice were euthanized by cervical dislocation and tissues were collected, weighed, immediately frozen on dry ice and stored at –80ºC.
Oral exposure was performed in one dose of 0.5 ml vanilla custard (of organic origin, Demeter, The Netherlands) to avoid discomfort due to oral gavage, see Chapter 5 for a picture. The compounds were dissolved in DMSO and added to the custard at a dose of 0.33 µl DMSO/gram bw. Four days before the actual exposure, mice were accustomed to eat 0.5 ml vanilla custard daily. All animals consumed their portion of 0.5 ml vanilla custard within 5 minutes. The period between oral exposure and sacrifice of the animals was 14 hours, six hours longer than after IP exposure to allow extra time for internal uptake.
The low and high exposure concentrations (Table 1) for EP and BPA were based on Lemmen et al. (*6, 7*). Since NP and BPA are more or less equipotent *in vitro* (*3*), the same dose was

chosen for NP. For the phthalates, concentrations were chosen based on maximal human exposure to DEHP in medical procedures, which was reported to be about 0.01-140 mg/kg bw (*8*). Based on these values a high dose of 100 mg/kg bw and a lower dose of 30 mg/kg bw, one log scale lower, were chosen. For DEHA the same concentrations as for DEHP were tested, as these two compounds are plasticizers used in a comparable way. The dose of *p,p'*-DDE was chosen based on the concentrations used in the study of You et al. (*9*), which dosed 10 and 100 mg/kg bw. The highest dose of quercetin was derived from the human intake with dietary supplements which can amount up to 1 gram/day (*10*), which is equivalent to 16.6 mg/kg bw/day for a 60 kg person. All concentrations were below levels of known toxicity and the same for both routes of exposures. The doses also were expressed as estradiol equivalents (EEQs) by multiplication with the *in vitro*-based estradiol equivalency factors (EEF) for the compounds (Table 1).

Table 1: The low and high dosages of estrogenic compounds ER-luc male mice received once, either orally in vanilla custard or IP in corn-oil. The high dosage of each compound also is expressed in Estradiol Equivalents (EEQ) using Estradiol Equivalency Factors (EEF) based on ERα-based in vitro studies. When available from the European Commission (EC) or the Food and Agricultural Organization (FAO), the Tolerable Daily Intake (TDI) for the compounds is given as well.

	Dose (mg/kg bw)		TDI (mg/kg bw)	EEF	EEQ high dose (mg/kg bw)	TDI, as EEQ (mg/kg bw)
	Low	High				
DMSO	0.33 µl/kg		-	0	0	-
EP	0.3	1	-	>1 (50)	>1	-
BPA	10	50	0.01 (51)	$2.5 \cdot 10^{-5}$ (3)	$1.3 \cdot 10^{-3}$	$2.5 \cdot 10^{-7}$
NP	10	50	-	$4.6 \cdot 10^{-5}$ (3)	$2.3 \cdot 10^{-3}$	-
p,p'-DDE	5	25	0.002 (52)	$2.3 \cdot 10^{-6}$ (22)	$5.7 \cdot 10^{-5}$	$1.1 \cdot 10^{-7}$
DEHP	30	100	0.05 (53, 54)	$2.2 \cdot 10^{-7}$ (3)	$2.2 \cdot 10^{-5}$	$1.1 \cdot 10^{-8}$
DEHA	30	100	0.3 (55)	$4.9 \cdot 10^{-8}$ (3)	$4.9 \cdot 10^{-6}$	$1.5 \cdot 10^{-8}$
DIHP	30	100	-	$3.1 \cdot 10^{-7}$ (3)	$3.1 \cdot 10^{-5}$	-
Quercetin	1.66	16.6	-	$2 \cdot 10^{-7}$ (10)	$3.3 \cdot 10^{-6}$	-

-: Not Available

Based on a pilot study with 3 animals per group exposed IP to EP it was decided not to collect lung, prostate, esophagus, heart and intestines as in these tissues no luc activity was found eight hours after IP exposure (data not shown), and the tissues analyzed in the present study are listed in Table 2.

Measurement of luciferase activity

Tissues were homogenized manually with an Eppendorf micro-pestle in 1.5 ml vials (Eppendorf, Hamburg, Germany) in lysis buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT and 1% Triton X100; pH 7.8). Livers were cut in 4 equal pieces of which three were analyzed, brain was cut in half, all other tissues were analyzed in full. The amount of lysis buffer added per tissue (Table 2) was sufficient to homogenize the sample

Table 2: Amount of lysis buffer added to the tissues before homogenization with micro-pestles and the fold dilution of the samples with PBS to prevent interference of Triton X100 in the protein measurement.

Tissue	µl Lysis buffer	Fold dilution in PBS
Pituitary	100	10
Brain per part	500	25
Tibia	200	10
Femur	200	10
Liver per part	500	100
Testis	500	10
Adrenal	100	10
Kidney	500	50

without diluting the luciferase activity too much. Tibia and femur were homogenized as good as possible with the micropestle. The samples were kept on ice during homogenization, after which the Eppendorf vials were centrifuged for 15 minutes at 13,000 g at 4ºC. After centrifugation the supernatant was quantitatively diluted in lysis buffer to approximately 1 mg/ml protein, 25 µl was pipetted in duplicate into a white 96 well assay plate (Costar, Corning, NY, USA). Light emission was measured at room temperature in a luminometer (Labsystems, Luminoskan RS) during two seconds, before and after automated addition of 100 µl 'flashmix' (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA·$2H_2O$, 2 mM DTT, 0.47 mM D-luciferin, 5 mM ATP; pH 7.8), subsequently light emission was extinguished with 50 µl 0.2 M NaOH before the next well was measured to prevent cross scattering of light.

Protein analysis

The protein content of the supernatant was determined using the Pierce protein BCA assay according to manufacturers' instructions (Pierce, Rockford, IL) with some minor modifications regarding pH, since in the present study pH of the samples was adjusted to 7.8 before analysis. The samples were diluted in PBS (Table 2) before protein analysis to prevent an excess of Triton X100, which would interfere with the Pierce protein BCA assay. Of this dilution 10 µl was analysed in duplicate in a 96 well flat bottom Greiner plate (Alphen a/d Rijn, The Netherlands). On each plate a full calibration curve from 0-1.5 mg/ml bovine serum albumin in Triton X100 in the same dilution in PBS was included in triplicate.

Data analysis

The relative light units (RLU) of the supernatant of all samples, prepared as described above, were corrected for the background RLU from the sample before addition of luciferin using MS Excel 2003. For comparison of the luc induction between tissues and animals, the luc activity was normalized for the protein level and expressed as relative light units (RLU)/ mg protein. Statistical analysis was performed with SPSS 12.0.1 for Windows. Differences

between tissues from control and exposed animals were tested for statistical significance using a Student's t-test ($p<0.05$). A dose-response analysis was performed by testing correlations between DMSO, low and high doses using the cross tabular statistics for correlations in SPSS 12.0.1. To be able to compare the luc inductions between tissues with different background luc activities induction factors (IF) were used. Induction factors were calculated using the formula: (RLU/mg protein $_{\text{average exposed}}$) / (RLU/mg $_{\text{average DMSO}}$).

Results

The mice were responsive to EP exposure and the luc activity (RLU/mg tissue) in the liver was more than 6000 RLU/mg protein upon dosing 1 mg/kg bw via both exposure routes (Table 3). The luc activity in the liver of the orally EP exposed mice, was almost four times higher than the luc activity in the livers of IP-exposed mice, while the orally DMSO exposed mice had a 2.7 times, but not significant, higher background luc activity in the liver compared to the IP DMSO exposed mice (Table 3).

Table 3: Luciferase activity expressed as Relative Light Units/mg protein (±SEM) in tissues from ER-luc transgenic male mice, 14 or 8 hours after respectively a single oral or IP dosage for mice exposed to the solvent control (DMSO) or the positive control dosed at 0.3 and 1 mg/kg bw (EP 0.3 and EP1).

Oral exposure	**DMSO (n = 9)**	**EP 0.3 (n = 8)**	**EP 1 (n = 8)**
Liver	0.8 ± 0.1	13,270 ± 9636	16,961 ± 4790*
Testis	20 ± 3.8	19± 3.4	44 ± 7.1@*
Kidney	0.3 ± 0.1	3.9 ± 1.0*	57 ± 20@*
Adrenal	0.1 ± 0	67 ± 34	440 ± 146@*
Brain	6.4 ± 1.6	8.2 ± 0.8*	16 ± 1.9@*
Pituitary	144 ± 35	586 ± 240	355 ± 248
Femur	0.5 ± 0.1	8.0 ± 4.3	34 ± 4.7@*
Tibia	1.7 ± 0.9	7.1 ± 1.1*	54 ± 2.7@*
IP exposure	**DMSO (n = 6)**	**EP 0.3 (n = 6)**	**EP 1 (n = 6)**
Liver	0.3 ± 0.1	3,324 ± 1058*	6,760 ± 2039*
Testis	17 ± 2.8	47 ± 12*	88 ± 13@*
Kidney	0.3 ± 0.1	116 ± 31*	332 ± 58@*
Adrenal	0.4 ± 0.2	132 ± 52*	277 ± 181
Brain	6.7 ± 0.5	12 ± 2.0	20 ± 3.3*
Pituitary	235 ± 84	746 ± 132*	441± 80
Femur	1.8 ± 1.7	84 ± 25*	148 ± 33*
Tibia	0.8 ± 0.3	66 ± 29*	121 ± 34*

n= the number of animals per exposure group.
*Indicates significant differences with the DMSO-exposed animals ($p<0.05$)
@ indicates significant difference between dosage groups ($p<0.05$)

Table 4: ER-mediated tissue specific induction factor (IF) of luciferase activity (Relative Light Units (RLU)/mg protein) relative to control induction in the same tissue 14 or 8 hours after respectively a single oral or IP dosage in male ER-Luc mice in the testis, kidney, tibia and femur.

Exposure	Testis		Kidney		Tibia		Femur	
	Oral	IP	Oral	IP	Oral	IP	Oral	IP
DMSO	1 ± 0.19	1 ± 0.17	1 ± 0.38	1 ± 0.26	1 ±0.54	1 ± 0.35	1 ± 0.17	1 ± 0.91
BPA 10 mg/kg	1.09 ± 0.22	0.92 ± 0.15	1.62 ± 1.08	1.84 ± 0.36	1.21 ± 0.5	2.64 ±1.39	1.43± 0.36	0.86 ± 0.19
BPA 50 mg/kg	0.72 ± 0.24	2.99 ± 0.6*	0.91 ± 0.42	322.36 ± 70.1*	1.27 ± 0.65	9.64 ± 2.14*	0.75 ± 0.23	4.89 ± 1.95
p,p'-DDE 5 mg/kg	0.98 ± 0.28	1.33 ± 0.23	0.79 ± 0.32	8.07 ± 6.66	0.24 ± 0.11	0.67 ± 0.35	0.67 ± 0.14	0.61 ± 0.39
p,p'-DDE 25 mg/kg	1.37 ± 0.44	1.11 ± 0.24	1.23 ± 0.32	10.21 ± 5.96	0.36 ± 0.13	1.07 ± 0.68	0.84 ± 0.19	0.48 ± 0.33
DEHA 30 mg/kg	0.94 ± 0.12	1.03 ± 0.19	0.78 ± 0.32	1.3 ± 0.74	0.51 ± 0.07	3.49 ± 1.73	0.83 ± 0.1	1.24 ± 0.64
DEHA 100 mg/kg	0.97 ± 0.2	0.95 ± 0.19	0.67 ± 0.29	10.61 ± 5.04	0.49 ± 0.09	1.39 ± 0.57	0.96 ± 0.16	0.69 ± 0.38
DEHP 30 mg/kg	1.79 ± 0.67	1.64 ± 0.3	0.64 ± 0.22	6.81 ± 3.52	1.85 ± 0.99	0.79 ± 0.6	0.73 ± 0.13	0.72 ± 0.64
DEHP 100 mg/kg	0.77 ± 0.1	0.57 ± 0.09*	0.94 ± 0.35	4.59 ± 2.6	0.47 ± 0.14	0.63 ± 0.36	1.05 ± 0.14	0.47 ± 0.25
DIHP 30 mg/kg	0.82 ± 0.14	1.13 ± 0.13	1.1 ± 0.47	0.93 ± 0.29	0.69 ± 0.24	2.78 ± 2.15	0.67 ± 0.13	0.79 ± 0.7
DIHP 100 mg/kg	1 ± 0.2	1.16 ± 0.21	0.73 ± 0.24	7.95 ± 7.05	0.2 ± 0.06	1.08 ± 0.5	0.91 ± 0.12	0.67 ± 0.54
NP 10 mg/kg	0.77 ± 0.17	1.43 ± 0.24	0.78 ± 0.41	8.68 ± 6.89	0.85 ± 0.13	0.38 ± 0.07	0.88 ± 0.14	0.3 ± 0.07
NP 50 mg/kg	1.09 ± 0.28	2.25 ± 0.76	1.17 ± 0.42	3.45 ± 1.21	0.37 ± 0.13	2.02 ± 1.35	0.78 ± 0.15	0.83 ± 0.48
Quercetin 1.66 mg/kg	1.57 ± 0.7	1.21 ± 0.21	1.58 ± 0.52	2.34 ± 1.61	0.79 ± 0.4	0.3 ± 0.08	1.01 ± 0.18	0.31 ± 0.14
Quercetin 16.6 mg/kg	0.98 ± 0.24	1.1 ± 0.14	8.07 ± 4.63	2.78 ± 1.5	1.01 ± 0.44	2.8 ± 1.48	0.66 ± 0.07	0.65 ± 0.45
EP 0.3 mg/kg	0.93 ± 0.17	2.82 ± 0.7*	11.53 ± 3.02*	370.7 ± 97.84*	4.14 ± 0.64*	84.96 ± 37.48*	16.73 ± 8.93	45.75 ± 13.85*

Average induction factors ± SEM are shown. * indicates significant differences from control (Student's t-test, $p<0.05$).

Tissues in which oral exposure to both concentrations of EP induced luc activity were: kidney, brain and tibia. Tissues in which only one of the two concentrations EP: induced luc activity after oral exposure were liver, testis, adrenal and the femur (Table 3).
After IP exposure both dosages EP induced luc activity in: liver, testis, kidney, femur and tibia. One of the two concentrations induced luc activity after IP exposure of EP in the adrenal, brain and pituitary (Table 3). Dose-related luc induction upon IP and oral exposure to EP was observed for liver, testis, kidney, adrenal, brain, femur and tibia (Table 3). Responses for pituitary, femur, tibia and adrenals (Table 3) have high standard deviations and are less often statistically significant than the other tissues. This probably was due to the very small tissue size or difficulty in homogenizing these tissues.

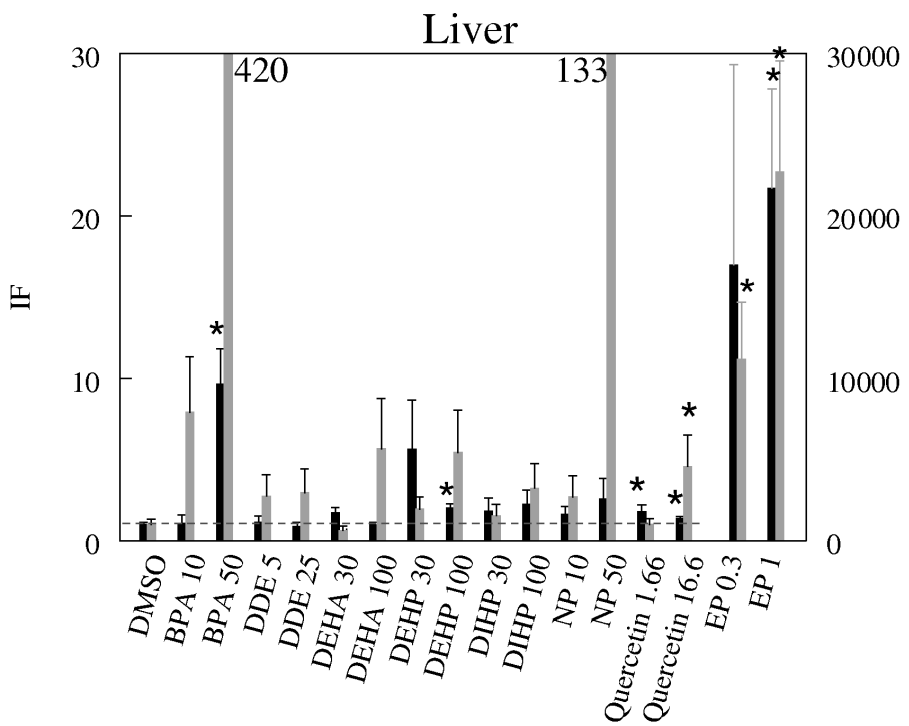

Figure 1: Graphic presentation of the ER-mediated tissue specific induction factor of luciferase activity (Relative Light Units (RLU)/mg protein) relative to control induction in the same tissue 14 or 8 hours after respectively a single oral (black) or IP dosage (grey) in male ER-Luc mice for the liver. Average induction factors ± SEM are shown. * indicates significant differences from control (Student's t-test, $p<0.05$). EP-bars use the right y-axis, 1000 times higher than the left y-axis. The dotted line indicates the luc induction factor (of 1) in control animals.

Table 4 presents the results obtained for ER-receptor mediated tissue specific induction of luciferase activity when mice were dosed orally or IP with the different food associated estrogenic compounds including data for testis, kidney, tibia and femur. The results obtained for the liver are presented in Figure 1. From the results it can be concluded that, especially in the kidney, and to a lower extent also in other tissues except for the liver, induction levels were higher upon IP than upon oral dosing (Table 4).
The induction factor (IF) in the liver of mice exposed to 1 mg/kg bw EP was more than 10,000 for both exposure routes (Figure 1). NP, *p,p'*-DDE, DEHA, DIHP and quercetin did not significantly induce luc activity in the testis, kidney, tibia or femur after oral and IP dosing (Table 4).
BPA induced luc activity in the liver (significant after oral exposure only), testis, kidney and tibia (after IP exposure only) in a dose-related manner which was statistically significant. DEHP induced luc activity in the liver after oral exposure and reduced the luc activity in the testis after IP exposure (Table 4). Quercetin significantly induced the luc activity in the liver after oral exposure to both concentrations and after IP exposure in the highest concentration only (Figure 1).

Discussion

The present study shows that some compounds known to be estrogenic *in vitro*, can be estrogenic *in vivo* as well, after oral as well as IP exposure. In the present study the maximum luc-induction was more than 20,000-fold in the liver, both upon a single IP and oral exposure to 1 mg EP/kg bw after 8 and 14 hours respectively (Table 3 and 4). This is within the same order of magnitude as the 10,000-fold induction reported by Lemmen *et al.* (*6*) observed 24 hours after IP exposure.
In most studies using estrogen receptor reporter gene mice, animals were dosed IP (*6, 7, 11, 12*) or subcutaneously (*13, 14*) both representing a worst case exposure scenario for food-associated compounds. This is suitable to test the relative toxico-dynamic potency of those compounds and their tissue-specific activity. However, for assessing the estrogenic risk of food-associated compounds, oral dosing is more relevant. In the present study, most compounds induced stronger responses after IP exposure compared to oral dosing (Table 4). A single high IP dose in the intraperitoneal cavity will result in a relatively high concentration of the parent compound in the blood, whereas after oral exposure the parent compound will be prone to first pass metabolism before reaching the circulation (*15*).
Relatively high background luc activities were observed in pituitary, testis and brain of control animals (Table 3). This probably reflects the high levels of ERs that have been reported in these tissues (*16, 17*).
Due to the persistent nature of *p,p'*-DDE, and because it is the major metabolite of DDT (*18*), this compound was chosen as one of our model compounds relevant for the human food chain. After IP exposure to *p,p*'-DDE there was a dose-related decrease but a non-significant trend in luc induction in testis, liver and kidney. In another study, 5 mg *p,p'*-DDE/kg bw induced luc activity in several tissues, such as liver, lung and heart after IP exposure (*11*). The estrogenicity of *p,p'*-DDE is debated, several studies reported its estrogenicity (*18, 19*), whereas others reported (anti-) androgenic activity (*20, 21*), which could indirectly

lead to estrogenic effects *in vivo*. Juvenile ER-mediated reporter gene fish exposed to the parent compound of *o,p'*-DDE, *o,p*'-DDT, showed a 1000 times higher *in vivo* estrogenicity compared to estradiol after 96 hours of exposure compared to the relative *in vitro* estrogenicity (*4*). This can be explained by the hydrophobic nature of *o,p'*-DDT which stimulates rapid bio-concentration by the fish in the aquatic environment. As *o,p'*-DDT is almost a thousands time more potent *in vivo* than *in vitro* (*4*), it was expected that this might be true for *p,p'*-DDE, with an *in vitro* relative induction compared to β-estradiol of $2.3 \cdot 10^{-6}$ (*22*). The results obtained in the present study support the need for more *in vivo* data.

In the present study NP did not induce any significant estrogenic effect, which is not in line with the estrogenicity detected *in vitro* (*3*), although a dose-related increasing trend was observed in the liver (Figure 1). This difference cannot be explained by the choice of the chemical NP-mixture used for exposure as the congener and purity used (85% of 4-n-nonylphenol from Fluka) is the estrogenic form (*23*). One plausible explanation would be the rapid clearance of NP *in vivo*. The half-life of NP after oral gavage has been reported to be maximal 3.5 hours (*24*) which is in accordance with the $t_{1/2}$ values reported by Green *et al.* (*25*). In addition to this, the uptake of NP after oral exposure has been reported to be low, in humans the bioavailability only was 20% (*26*). Only after repeated doses up to 1 g/kg bw NP has been shown to be estrogenic in rodents (*27*). Taking into account only experiments with non-toxic doses of NP thereby excluding nephrotoxicity (*28*) and weight loss (*29, 30*), some estrogenic effects were reported. For example ovariectomized prepubertal Long-Evans and Noble rats dosed for three days either orally or subcutaneously with 75-200 mg NP/kg bw/day, had over 100% increased uterine wet weights. In pre-pubertal Long-Evans rats, the highest increase in uterine wet weight was seen after oral dosing compared to subcutaneous dosing (*31*). The age at vaginal opening was decreased significantly with 5.3 and 6.8 days after exposure to 50 and 100 mg NP/kg bw respectively starting from PND21 to PND35, effects on body weight were lacking (*31*). Increased uterine weights also were observed in female CD-1 mice exposed subcutaneously from postnatal day 17 till 20 to NP to 10 mg/kg bw and higher (200%) increase based on uterine weight/body weight ratio (*27*). In a three-generational experiment with Sprague-Dawley rats the absolute ovary weight in the second generation was decreased with 13.7 and 20.7% in the high dose groups (more or less equivalent to 30-100 and 100-350 mg/kg bw/day), however when corrected for body weight, these changes were both around 12% (*29*). These results suggest that NP is estrogenic *in vivo*, only after repeated high doses during a longer time, probably because of its fast degradation. Even in our experiment where luc induction is a quite sensitive biomarker for ER-activation, NP was not able to significantly induce luc activity after single dosing (Figure 1, Table 4). This is in accordance with the lack of induction reported from an *in vivo* experiment with transgenic ER-mediated reporter gene zebrafish continuously exposed to nominal water concentrations up to 1 µM NP during 96 hours of exposure (*4*). When exposure was longer and to very high concentrations, amounting up to 3 weeks and 2.27 µM NP, vitellogenin, a biomarker for estrogenic exposure, was induced in zebrafish (*32*).

In *in vitro* studies DIHP was a weak estrogenic compound (*3, 33*). The reported estrogenicity of DIHP, however, is so low (EEF of $3 \cdot 10^{-7}$, Table 1) that the effect concentrations are close to the solubility limits. Our present results are in accordance with other *in vivo* studies where DIHP was estrogenic in extremely high doses. For example ovariectomized Sprague-Dawley

rats exposed via oral gavage to 200 and 2000 mg/kg bw for four consecutive days had significantly increased uterine wet weights. This effect, however, could not be reproduced in later experiments by the same authors (*33*). As with *p,p'*-DDE, the estrogenicity of DIHP is under debate and the results obtained in *in vitro* studies on the estrogenicity of DIHP are not consistent. DIHP was estrogenic in Chinese Hamster Ovary (CHO) and in human osteoblastic (U2-OS) ER reporter gene cell lines (*3, 34*), but not in ER ligand-binding assays performed with immature Sprague-Dawley uterine cytosol and in human reporter gene assays in MCF-7 and HeLa cell lines (*33*). Due to the nature of DIHP, being estrogenic *in vivo* and *in vitro* only at very high concentrations (*3, 35*), it is not surprising that DIHP only showed a slight non-significant trend in luc induction in ER-luc mice.

The slightly more estrogenic phthalate, DEHP significantly induced luc activity in the liver with a factor of 2 in the highest dosage group (Figure 1) and reduced the luc activity in the testis at the high dose (Table 4). The estrogenicity of DEHP was shown before, with an induction of the uterine wet weight of 54% after a single oral gavage of 2 g/kg bw DEHP in ovariectomized Sprague-Dawley rats. This effect, however, was not reproducible in a second experiment with 15-22% heavier rats as had been the case for DIHP (*33*). The significant 2-fold reduction of luc activity compared to control in the testis in the DEHP high dose group might be explained by the anti-androgenicity (*36*). Phthalates have been shown to be either estrogenic or anti-androgenic and are rapidly metabolized both in experimental animals as well as in humans (*37*). More effects are therefore to be expected upon chronic exposure to high concentrations.

BPA induced luc activity in the liver after oral exposure (Figure 1), and in the testis, kidney and tibia after IP dosage (Table 4). In pregnant females BPA induced an estrogenic response in fetuses 2 hours after a single maternal IP exposure of 1 mg/kg bw on day 13.5 post coitum (*7*). A lower dose up to 0.2 mg BPA/kg bw/day via oral gavage or subcutaneously for at least 6 subsequent days given to male C57black/6N mice, did not have an effect on the male reproductive organs irrespective of the life stage they were exposed in (*38*). In adult C57black ER reporter gene mice, luc activity was significantly induced in the uterus 20-24 hours after a single subcutaneous injection with doses between 0.8-25 mg BPA/kg bw, whereas the uterine wet weight was only slightly significantly increased (*39*). This was to be expected as gene induction usually is a much faster response, within hours, compared to a physiological response such as uterine weight, which takes three days (*40*). When BPA was administered in the diet, intra-peritoneally or subcutaneous in levels up to almost 1 g/kg bw/day up to 68 days to Wistar, Holtzman SD rats and CD-1 and C57black mice effects were mainly present in rats, not in mice. A significant increase of sterile seminiferous tubules, causing a decrease in prostate and preputial weight, was seen after oral and subcutaneous dosing of 20 mg - 1 g BPA/kg bw/day for at least 28 days (*41*). The relative weak estrogenic effects of BPA *in vivo* compared to *in vitro* could be due to its efficient metabolic clearance. Already 1.5 hours after oral administration of 100 mg BPA/kg bw maximum blood concentration of BPA were reached in DA/Han rats. After 24 hours BPA was not detectable in the blood anymore (*42*). In humans, BPA is extensively glucuronidated and the BPA-glucuronide is excreted in urine (*43*). From our results (Table 4) it is clear that an oral dose of BPA usually results in much lower luc induction than an IP dose (Table 4). This is in accordance with results obtained in Fischer 344 rats, in which IP dosing resulted in a 2 times higher maximal

blood concentration after dosing for seven consecutive days (*44*). In the same study, the resulting blood concentration of BPA given IP was up to 164 times greater compared to blood BPA levels in orally dosed animals. In rats and monkeys, the toxicokinetics of BPA was not comparable. After oral administration of 10 mg BPA/kg bw the area under the curve in blood of in cynomolgus monkeys was 20 times higher compared to that in rats (*45*). This might indicate that the responses in rodents could be an underestimation of the effects in humans. On the contrary, if the effects would be related to the plasma concentration of free BPA, rather than to the total concentration of BPA (free and conjugated), the results obtained in rodents may be an overestimation of the effects to be expected in humans since plasma levels of free BPA in rats might be higher than in humans (*46*).

Quercetin, a major flavonoid present in food, induced estrogenic effects in the liver upon oral exposure of ER-luc mice. The highest non-significant luc induction (8.1 times) was seen in the kidney after oral exposure (Table 4). Previously De Boer *et al.* (*47*) studied the tissue distribution of quercetin in rats and demonstrated significant quercetin tissue concentrations in especially the lungs but also in the kidney and testes (*47*). The induction in the liver was significant for both doses via oral exposure (Figure 1). The *in vitro* results already showed that quercetin was estrogenic both via the ERα as well as the ERβ in human osteoblastic reporter gene cells. The EEFs were respectively $2 \cdot 10^{-7}$ and $7 \cdot 10^{-6}$ and supra-maximal inductions were observed relative to the maximum response of E_2 of 170% (via ERα) and up to 450% (ERβ) (*10*). Quercetin also induced cell proliferation in a human cervix epitheloid carcinoma cell line (HeLa) transfected with an ERα expression vector, whereas proliferation was absent in the original cells without ERα, the proliferation thus likely being caused by estrogenic action (*48*). Although in the ER-luc mice no distinction can be made between induction via the ERα or the ERβ, another report mentions that quercetin might probably induce the ERβ (49), the current study is the first to prove that quercetin is estrogenic *in vivo* in male ER-luc reporter gene mice.

The lowest oral exposures that induced significant estrogenic response in the liver of the ER-luc male mice was $3.3 \cdot 10^{-6}$ mg EEQ/kg bw (calculated based on *in vitro* EEFs, Table 1). In the real-life situation, however, intakes of estrogenic compounds are chronic. This can very well result in effective internal concentrations of especially more persistent compounds. The tolerable daily intake (TDI) for the estrogenic compounds should protect consumers from this. The TDI's for the tested compounds expressed as EEQ are in the range of $1.1 \cdot 10^{-8}$ - $2.5 \cdot 10^{-7}$ mg/kg bw (Table 1), which is about $10\text{-}10^4$ times lower than the lowest effect levels, expressed in EEQ's, in this study. For BPA, for example, the no observed effect level for luc induction in the liver upon oral intake was 10 mg/kg bw/day and the lowest observed effect level was 50 mg/kg bw/day, with a TDI of 0.01 mg/kg bw/day the margin between the lowest effect level and the TDI is 5000.

The current study reveals that biomarker-responses in adult mice after a single oral exposure occur at exposure levels $10\text{-}10^4$ times higher than the established TDI's for some of these estrogenic compounds. Given the facts that i) the present study did not include chronic exposure and that ii) simultaneous exposure to multiple estrogenic compounds may be a realistic exposure scenario, it remains to be seen whether this margin is sufficiently high.

Abbreviations:

p,p'-DDE, 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene; EC_{50}, the concentration that induces 50% of the maximal response; EEF, estradiol equivalency factor; EEQ, estradiol equivalent; ER, estrogen receptor; ER-Luc mice, Estrogen Receptor reporter gene (luciferase) mice; IF, Induction Factor; IP, intraperitoneal; RLU, relative light units; TDI, Tolerable Daily Intake.

Keywords:

estrogen receptor, reporter gene mice, endocrine disrupter, phthalates, bisphenol A, nonylphenol, *p,p'*-DDE, food contaminants.

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Chapter 4:

Estrogenicity of food associated estrogenic compounds in the offspring of female estrogen receptor-reporter gene mice upon oral and IP maternal exposure.

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4

Abstract

The present study investigated to what extent seven food associated *in vitro* estrogenic compounds can induce estrogenic effects in the fetuses of pregnant female mice with an estrogen receptor (ER)-mediated luciferase (luc) reporter gene system. The luc induction was determined eight hours after a single IP dose or after dosing for eight days orally. Three known estrogens, 17β-estradiol (E_2), 17α-ethynylestradiol (EE) and 17β-estradiol 3,17-dipropionate (EP) were used as positive controls at 1 mg/kg bw and DMSO as solvent control. The food associated estrogenic compounds tested were: bisphenol A (BPA), nonylphenol (NP) both at 50 mg/kg bw, dichlorodiphenyldichloroethylene (*p,p'*-DDE) at 50 mg/kg bw, quercetin at 16.6 mg/kg bw, and di-isoheptyl phthalate (DIHP), di-(2-ethylhexyl) phthalate (DEHP) and di-(2-ethylhexyl) adipate (DEHA) all at 100 mg/kg bw. Exposure to E_2, EE and EP resulted in significant luc inductions upon both oral and/or IP dosing in a variety of tissues including liver, tibia and femurs, and upon IP dosing also in fetuses.
BPA, NP, DEHA, DEHP, DIHP, DDE and quercetin were unable to significantly induce luc activity in fetuses. However, after oral exposure to NP, BPA and DIHP placental luc activity was significantly lowered.
The results indicate that at the current levels of exposure to food associated estrogenic compounds, estrogenic effects to the fetus are not expected. The significant luc reduction in the placenta, should be further studied for its significance for fetal development and relevance for the human situation.

Introduction

Estrogenic compounds are ubiquitously present in the food chain. Well known phyto-estrogens are genistein and quercetin, present in fruits and vegetables (*1,2*). These phyto-estrogens were recently proven to be estrogenic *in vitro* in a human osteoblastic transgenic cell line containing either one of the two estrogen receptors (ERs); ERα or ERβ (*3*). Next to phyto-estrogens present in food, pseudo-estrogens are present in food packaging, such as plastic wraps and tin coatings (*4,5,6*). Well-known examples of estrogenic compounds in food packaging are bisphenol A (BPA), nonylphenol (NP) and several phthalates. In our laboratory several other food associated compounds have been shown to be estrogenic *in vitro* as well (*6*). BPA has been shown to be estrogenic *in vivo* as well in estrogen receptor luciferase reporter gene (ER-luc) female mice 8h after IP exposure but this effect was no longer observed 24h after IP exposure (*7,8*). In this study the IP-dosed DES and BPA induced a time-dependent luc-activity in the fetuses of the pregnant female mice. This demonstrates the usefulness of the model to detect transfer of estrogens to fetuses of IP-exposed pregnant ER-reporter gene female mice already after 8 h of exposure (*7,8*). The effects of DES still were measurable after 24h as well. The study was performed using IP-exposure which leads to high dosage peaks in the blood plasma, and not using the more realistic oral exposure that has been shown to result in much lower responses in Luc-male mice before (*9*). Very early (fetal) life stages are generally considered to be more susceptible to exposure to toxicants because of the relatively low levels of defence mechanisms against xenobiotics, such as metabolic enzymes (*10*), and the relatively fast growth and development of various tissues. Maternal exposure during pregnancy might lead to fetal exposure, as was shown for BPA and DES in rodents (*8,11*). In the human situation, DES is a well-known example of a compound for which maternal exposure leading to fetal exposure causes adverse effects later in life (*12*).

The aim of the present study was to investigate whether *in vivo* estrogenic effects during critical fetal life stages can be expected upon maternal oral exposure to food associated estrogenic compounds. Organogenesis, the crucial period of development, starts with the formation of the ecto-, meso- and endoderm (early organogenesis) from gestation day (GD)5-10 and continues until GD14 in rodents (*10*). Pregnant ER-luc mice were dosed orally for eight days with the food associated model compounds, to resemble continuous exposure throughout the main period of organogenesis. To be able to compare the findings of the present study with those obtained in previous studies, the effect of single peak exposure via IP dosing was studied as well.

The model compounds tested in the present study included three known estrogens 17β-estradiol (E_2), 17α-ethynylestradiol (EE) and 17β-estradiol 3,17-dipropionate (EP) used as positive controls and seven food-associated estrogenic compounds including bisphenol A (BPA), nonylphenol (NP), dichlorodiphenyldichloroethylene (*p,p'*-DDE), quercetin, di-isoheptyl phthalate (DIHP), di-(2-ethylhexyl) phthalate (DEHP) and di-(2-ethylhexyl) adipate (DEHA).

The *in vivo* estrogenicity of these food associated compounds was characterised in tissues of pregnant ER-luc reporter gene mice and in the fetuses after repeated oral as well as single IP maternal exposure.

Animals, Materials and Methods

Chemicals and reagents

17β-Estradiol (E_2) (>98%, CAS: 50-28-2), 17β-estradiol 3,17-dipropionate (EP) (>98%, CAS: 113-38-2), 17α-ethynylestradiol (EE) (>98%, CAS: 27-63-6), *para, para* -dichlorodiphenyldichloroethylene (*p,p'*-DDE) (>99%, CAS: 72-55-9), bisphenol A (BPA) (>99%, CAS: 80-05-7) were obtained from Sigma (Zwijndrecht, The Netherlands), quercetin dihydrate (99%, CAS: 6151-25-3) was obtained from Acros Organics (Geel, Belgium), nonylphenol (NP) (~85%; technical mixture, CAS: 104-40-5) was obtained from Fluka (Buchs, Germany), di-(2-ethylhexyl) phthalate (DEHP) (CAS: 117-81-7) was obtained from TCI (Tokyo, Japan), di-isoheptyl phthalate (DIHP) (CAS: 41451-28-9) and di-(2-ethylhexyl) adipate (DEHA) (CAS: 103-23-1) were technical grade and obtained from Agrotechnology and Food Innovations BV, Wageningen.

Tricine (>99%), glycylglycine, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetra-acetic acid (EGTA), triton X-100, bovine serum albumin (BSA, >97%) and corn-oil were purchased from Sigma (Zwijndrecht, The Netherlands). Sodium hydroxide (NaOH, >99%), $EDTA{\cdot}2H_2O$ (>99%), $MgSO_4{\cdot}7H_2O$ (>99,5%) and 1,4-dithiothreitol (DTT) (>99%) were obtained from Merck (Darmstadt, Germany). D-luciferin (>99,5%) was obtained from Duchefa (Haarlem, The Netherlands). $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$ was obtained from Aldrich (Milwaukee, WI). ATP was obtained from Roche (Mannheim, Germany). Dimethyl sulfoxide (DMSO, >99,9%) was obtained from Acros Organics (Geel, Belgium).

Animals and experimental set-up

Of the ER-luc mice originating from the stable reporter line INS7 (*7,8*), 110 female mice were randomly bred within our colony. The mice were fed a phyto-estrogen free diet throughout their life (AM-II diet, Hope Farms, The Netherlands). Water was given in glass bottles, and water and food were provided *ad libitum*. The animal experiment was approved by the Animal ethics committee of Wageningen University.

Males and females were acclimatized for three weeks in separate animal rooms in order to synchronize the estrous cycle of the females, known as the "Whitten" effect (*13*). After the acclimatization period the mice were transferred to a Macrolon cage existing of two compartments. Males were kept in the same cage, but in a separate compartment next to the females for two days. After two days the compartments were united before darkness. Twelve hours later, females were checked for a vaginal plug twice daily for three consecutive days. The day mice had a vaginal plug was designated gestation day 0 (GD0).

Five females were used per group for oral exposure, (except for the 50 mg/kg bw BPA, 50 mg/kg bw NP, 100 mg/kg bw DEHA and 16,6 mg/kg bw quercetin which consisted of 4 mice), and five animals per group for IP exposure (except for the 100 mg/kg bw DIHP, 16,6 mg/kg bw quercetin and 1 mg/kg bw E_2 which consisted of 4 mice).

For intraperitoneal (IP) exposure DSMO stocks of the compounds were diluted to 5% in corn-oil, and 6.66 µl/g bw of this solution was injected IP on GD14. Eight hours after IP exposure, the mice were euthanized by cervical dislocation and fetuses, placentas and maternal organs were collected separately. All tissues were immediately frozen on dry ice and stored at -80ºC until analysis.

Oral exposure was performed in 0.5 ml vanilla custard to avoid discomfort from oral gavage, see Chapter 5 for a picture. Four days before the actual exposure (GD4 up to and including GD7), mice were accustomed to eat vanilla custard (of organic origin, Demeter, The Netherlands). Oral exposure was performed for 8 days from GD8 to GD15 by adding the desired amount of a test compound in 0.33 µl DMSO/g bw to the vanilla custard. The mice always immediately finished their custard. On GD16 mice were euthanized by cervical dislocation, fetuses, placentas and maternal organs (Table 2) were collected. All tissues were immediately frozen on dry ice and stored at -80ºC until analysis.

Table 1: The low and high dosages of estrogenic compounds ER-luc female mice received orally in vanilla custard (daily from GD8-15) or IP in corn-oil (once for eight hours on GD14). The dosages of each compound is also expressed as Estradiol Equivalents (EEQ) using Estradiol Equivalence Factors (EEF) based on ERα-based in vitro studies referred to in the footnotes.

Compound	Dose (mg/kg bw/day)	EEF	References	EEQ dose (mg/kg bw/day)
DMSO	0	0		0
E_2	1	1		1
EE	1	1.2	(21)	1.2
EP	1	6.5	(7,8,21)	6.5
BPA	50	$2.5 \cdot 10^{-5}$	(6)	$1.3 \cdot 10^{-3}$
NP	50	$4.6 \cdot 10^{-5}$	(6)	$2.3 \cdot 10^{-3}$
DDE	50	$2.3 \cdot 10^{-6}$	(40)	$1.2 \cdot 10^{-4}$
DEHP	100	$2.2 \cdot 10^{-7}$	(6)	$2.2 \cdot 10^{-5}$
DEHA	100	$4.9 \cdot 10^{-8}$	(6)	$4.9 \cdot 10^{-6}$
DIHP	100	$3.1 \cdot 10^{-7}$	(6)	$3.1 \cdot 10^{-5}$
Quercetin	16.6	$2 \cdot 10^{-7}$	(3)	$3.3 \cdot 10^{-6}$

Table 2: Amount of lysis buffer added to the tissues before homogenization with a micro-pestle and the fold dilution of the samples with PBS to prevent interference of triton X100 in the protein measurement.

Tissue	µl Lysis buffer	Fold dilution in PBS
Pituitary	100	10
Brain per part	500	25
Tibia	200	10
Femur	200	10
Liver	500	100
Fetus	500	100
Placenta	500	50

Table 1 presents the actual doses of the different compounds that were given to the animals either daily orally for 8 days or by a single IP dose. The concentrations used were chosen based on available information from literature as well as on previous studies in our laboratory in the following way. The dosages for EP and BPA were based on Lemmen *et al.* (*8*), in which 1 mg/kg bw induced a significant luciferase response in the fetuses 8 and 24h after IP-exposure. IP-exposure to 1 mg BPA/kg bw induced the highest luc-response in the fetuses after 8 hours, and the maximum induction for EP was reached after 24h with 10 mg EP/kg bw, and after 24h with 1 mg DES/kg bw (*8*). Since NP and BPA are roughly equipotent *in vitro* (*6*), the dosage that was previously shown to be effective for BPA (*9*) was chosen for NP as well. For the same reason the dosage for the hormones E_2 and EE, which are roughly equipotent to EP, were set at the same level as the dose used for EP. For the phthalates, concentrations were chosen based on maximal human exposure to DEHP in medical procedures (*14*) and the fact that at dosages higher than 100 mg/kg bw severe carcinogenic effects were reported (*15*), which should be avoided. DEHA is used in the same materials as DEHP and therefore the same concentrations were tested. The dose of *p,p'*-DDE was chosen based on the low dose used in the study by You *et al.*(*16*). The dose of quercetin was based on the human intake of quercetin dietary supplements which can amount to up to 1 g/day (*17*). For a 60 kg person this would be 16.6 mg/kg bw/day. Concentrations were chosen to be non-toxic, and were the same for both routes of exposure. The dosages also were expressed as Estradiol Equivalents (EEQ) by multiplying the dosages with the *in vitro*-based relative Estradiol Equivalence Factors (EEF) of the compounds (Table 1).

Protein analysis

Tissues were homogenized manually with an Eppendorf micro-pestle in 1.5 ml Eppendorf vials (Eppendorf, Hamburg, Germany) in lysis buffer (25 mM glycylglycine, 15mM $MgSO_4$, 4 mM EGTA, 1 mM DTT and 1% triton X100; pH 7.8). Livers were cut in 4 equal pieces of which three were analyzed as triplicates, the fourth piece was used as a back-up. Brains were cut in half and analyzed as duplicates. All other tissues and fetuses were analyzed completely at once. In short, samples were stored at -80ºC, defrosted on ice and lysis buffer was added before manual homogenization. The amount of lysis buffer added per tissue (part) is given in Table 2. After homogenization, vials were centrifuged for 15 minutes at 13.000 g at 4ºC. After centrifugation the supernatant was collected and its protein concentration determined using the Pierce protein BCA assay according to manufacturers' instructions (Pierce, Rockford, IL) with some minor modifications regarding pH, since in the present study the pH of the samples was adjusted to 7.8 before analysis. The supernatant of the samples was diluted in PBS (dilution folds are presented in Table 2) prior to analysis to prevent an excess of triton X100 which interferes with the Pierce protein BCA assay (Pierce, BCA Instruction manual). Of this dilution 10 µl was measured in duplicate in a 96 well flat bottom Greiner plate (Alphen a/d Rijn, The Netherlands). On each plate a full calibration curve from 0-1.5 mg/ml bovine serum albumin in triton X100 in the same dilution in PBS was included in triplicate.

Luciferase activity measurement

Samples were quantitatively diluted in lysis buffer to approximately 1 mg/ml protein to prevent effects of large differences of protein content during the luc measurement. Of these

diluted samples 25 µl was pipetted in duplicate into a white 96 well assay plate (Costar, Corning, NY, USA). Light emission was measured at room temperature in a luminometer (Labsystems, Luminoskan RS) during two seconds, before and immediately after automated addition of 100 µl 'flashmix' (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA·$2H_2O$, 2 mM DTT, 0.47 mM D-luciferin, 5 mM ATP; pH 7.8). Subsequently light emission was extinguished with 50 µl 0.2 M NaOH before the next well was measured to prevent cross scattering of light.

Data analysis

The light output (relative light units, RLU) of the sample was corrected for the background RLU (measured before addition of luciferin). For comparison of the induction between tissues and animals, the luc activity was normalized for protein content and expressed as relative light units (RLU)/mg protein. To be able to compare the luc induction between tissues with different background luc activity, induction factors (IF) were used. Induction factors were calculated using the formula: (RLU/mg protein $_{\text{average exposed}}$) / (RLU/mg protein $_{\text{average DMSO}}$).
Statistical analysis was performed with SPSS 12.0.1 for Windows. Before analyzing differences between IP and oral exposure, a visual check for normality of the data was performed using histograms. If data were distributed normally, means were compared using a Student's t-test. When data were not normally distributed, the samples were analyzed using the Wilcoxon signed-rank test.

4

Results

Table 3 presents the luciferase activity expressed as Relative Light Units/mg protein (±SEM) in tissues of pregnant ER-luc mice and their fetuses, exposed orally for eight days in vanilla custard, or eight hours after a single IP dose in corn oil to the solvent control (DMSO) or the positive controls EP, E_2 and EE. Generally tissues showed higher luc inductions when mice were exposed IP, compared to oral exposure, except for the liver of mice exposed to the estrogens E_2, EE and EP for which oral dosing resulted in higher luc activity.
The background signal of the solvent control in the ER-luc mice ranged from 4.4 and 4.8 RLU/mg protein in the fetus and brain of the oral dosage group up to 3,616 RLU/mg protein in the placenta of the IP dosage group (Table 3). The difference between these two extremes in background luc activity is 821 fold. All three positive control estrogens E_2, EP and EE, induced luc activity in various tissues including brains, tibia and femurs upon both oral and IP exposure (Table 3). The liver did show a strong increase in luciferase activity, though non-significant due to the relatively high experimental variation (Table 3). Interestingly, the placenta did not respond to oral nor IP exposure to any of the estrogenic model compounds (Table 3). However, oral exposure of the pregnant mice to EE at 1 mg/kg bw/day for eight days resulted in resorption of the fetuses which is a remarkable difference with the effects induced by oral exposure to EP or E_2 which did not result in luc induction in the fetuses nor in resorption. IP exposure to the model estrogens induced luc activity in the fetuses, for all three estrogens, being the strongest by EE, while in contrast to oral exposure to EE, after IP exposure to EE no resorptions were observed (Table 3). For comparison Table 4 also presents the induction factors, as derived from RLU/mg protein presented in Table 3 for the estrogenic model compounds.

Table 3: Luciferase activity expressed as Relative Light Units/mg protein (±SEM) in tissues of pregnant ER-luc mice, 24 hrs after the last of 8 daily oral exposures or 8 hours after a single IP dosage to the solvent control (DMSO), the positive control (EP), E_2 and EE. n=the number of exposed animals per group, * indicates significant differences from control (Student's t-test, $p<0.05$).

Oral exposure (0.33 µl DMSO / g bw in 0.5 ml vanilla custard)	**DMSO** (n = 5)	**EP** 1 mg/kg/day (n = 5)	**E_2** 1 mg/kg/day (n = 5)	**EE** 1 mg/kg/day (n = 5)
Brain	4.8±0.8	7.7±2.2	12±3*	32±6*
Liver	31±19	291,905±284,257	242,700±211,707	13,021±10,957
Tibia	14±6	91±39	221±31*	1,369±441*
Femur	12±2	105±52	238±60*	1,188±317*
Placenta	647±47	524±44	692±54	#
Fetus	4.4±0.9	4.3±0.9	3.9±0.7	#
IP exposure (6.66 µl oil / g bw, 5% DMSO)	**DMSO** (n = 5)	**EP** 1 mg/kg/day (n = 5)	**E_2** 1 mg/kg/day (n = 4)	**EE** 1 mg/kg/day (n = 5)
Brain	8.7±1.2	22±7	17±3	27±7
Liver	47±24	6,875±1,718*	716±698	1,061±670
Tibia	16±2.2	341±60*	220±30*	282±56*
Femur	15±3.1	345±28*	227±36*	374±73*
Placenta	3,617±176	4,060±224	3,312±199	3,914±222
Fetus	9.1±1.1	16±2.0*	57±9*	142±14*

#: EE exposed at 1 mg/kg/day for eight days orally resulted in abortion

Table 4 presents the luciferase induction, derived from RLU/mg protein, for the responsive tissues: liver, brain, tibia, femur, and the placenta and fetus in pregnant ER-luc mice. Pituitary was not responsive and therefore not included. Although in the liver of the BPA and NP exposure groups luc induction was 45-67 fold compared to the control, the luc activity was not significantly induced in any of the maternal tissues.

Interestingly, oral exposure to the food associated model compounds, NP, BPA and DIHP significantly lowered instead of increased the luc activity in the placenta to 0.7, 0.7 and 0.6 times the respective solvent control values (Table 4). Oral exposure to DEHA significantly induced the luc activity in the placenta 1.2-fold (Table 4). After IP exposure NP, BPA, DIHP and also DEHA also significantly lowered the luc activity in the placenta respectively 0.7, 0.8, 0.8 and 0.8 times compared to the solvent control values (Table 4). In addition, NP and DEHA significantly lowered the luc activity in the fetus after IP exposure only (Table 4).

Table 4: ER-mediated tissue specific luciferase activity induction factors (IF) (relative to control induction) in the same tissue 24 hrs after the last of 8 daily oral exposures or 8 hours after a single IP dosage in female ER-Luc mice in the liver, brain, tibia, femur, fetus and placenta. Average induction factors ± SEM are shown. * Indicates significant differences from control (Student's t-test, $p<0.05$). @: due to resorptions no data available; β: data lost due to technical failure.

	Liver		Brain		Tibia		Femur		Fetus		Placenta	
Exposure	**Oral**	**IP**	**Oral**	**IP**	**Oral**	**IP**	**Oral**	**IP**	**Oral**	**IP**	**Oral**	**IP**
DMSO	1.0±0.6	1.0±0.5	1.0±0.2	1.0±0.1	1.0±0.4	1.0±0.1	1.0±0.2	1.0±0.2	1.0±0.2	1.0±0.1	1.0±0.1	1.0±0.0
BPA50	0.2±0.1	67±41	1.1±0.5	1.1±0.2	0.9±0.3	2.0±0.7	2.1±1.2	1.6±0.5	1.3±0.2	1.2±0.2	0.7±0.1*	0.8±0.1*
NP50	0.4±0.1	46±33	0.8±0.2	1.1±0.3	0.5±0.1	2.7±0.7	0.8±0.1	1.4±0.4	0.8±0.1	0.7±0.1*	0.7±0.1*	0.7±0.1*
DEHA100	0.1±0.0	34±17	0.6±0.2	1.1±0.4	0.5±0.1	0.9±0.2	0.6±0.1	0.9±0.2	0.9±0.2	0.6±0.1*	1.2±0.1*	0.8±0.0*
DEHP100	0.0±0.0	0.0±0.0	0.7±0.1	1.2±0.3	0.9±0.2	2.1±0.6	1.1±0.2	1.3±0.6	1.3±0.2	0.7±0.1	0.8±0.1	1.0±0.1
DIHP100	0.2±0.1	13±12	0.7±0.2	1.3±0.3	1.7±0.9	1.3±0.5	0.7±0.2	1.1±0.3	1.5±0.2	1.2±0.3	0.6±0.1*	0.8±0.1*
DDE50	0.4±0.1	4.1±2.3	0.6±0.1	0.8±0.1	0.6±0.1	0.7±0.1	0.7±0.1	0.7±0.1	1.1±0.1	0.8±0.1	0.9±0.1	1.1±0.1
Quercetin16.6	0.1±0.0	β	1.4±0.4	1.0±0.2	0.6±0.1	1.1±0.2	0.7±0.1	0.7±0.1	1.3±0.2	0.8±0.1	1.2±0.1	0.9±0.0
E_2	7,733±6746	15±15	2.5±0.7*	2.0±0.4	16±2.2*	14±2*	21±5*	15±2.3*	0.9±0.2	6.3±0.9*	1.1±0.1	0.9±0.1
EE	415±349	23±14	6.6±1.2*	3.1±0.8	96±31*	17±3*	103±27*	24±5*	@	16±2*	@	1.1±0.1
EP	9,301±9,058	148±37*	1.6±0.5	2.6±0.8	6.4±2.7	21±4*	9.1±4.6	23±2*	1.0±0.2	1.8±0.2*	0.8±0.1	1.1±0.1

Discussion

The aim of the present study was to investigate whether *in vivo* estrogenic effects during critical fetal life stages can be expected upon maternal exposure to food associated estrogenic compounds. To this end possible placental transfer during organogenesis, the crucial period of development, of estrogenic compounds was determined based on induction of luc activity in fetuses of pregnant ER-luc reporter gene mice. In addition to IP exposure the mice also were exposed orally in order to mimic the human exposure route to pseudo-estrogens via the food. The present study applied an animal friendly way to dose animals orally using vanilla custard which they always ate completely within a few minutes. At GD 16 the mice were sacrificed which was 24h after the last of 8 daily oral exposures, thus allowing additional time for uptake as compared to the 8h used after the single IP-exposure (*18*).

The average background luc activity in the liver after IP exposure to the DMSO solvent control in female mice was 47 RLU/mg protein (Table 3) which is 157 times higher compared to our previous study in which male ER-luc mice were dosed in the same way (*9*). These and similar gender-dependent differences that were observed for other organs (but the brain) can be explained by the fact that females generally have higher endogenous levels of the female hormone estradiol (*19*). Within the female mice of the present study also large differences in background luc activity values were observed between the different tissues, for example in the placenta the luc activity was over 100 times higher compared to that in the brain (Table 3).

In the present study IP-exposure to E_2 and EP significantly induced the luc activity in the fetuses compared to the control (Table 4). This is in accordance with the results of the study of Lemmen (*8*) where 8h after IP exposure to 0.1 mg DES/kg bw or 10 mg EP/kg bw luc activity was significantly induced in the fetuses. In the study of Lemmen *et al.*, IP-exposure to 1 or 10 mg BPA/kg bw significantly induced luc activity in the fetuses. This effect was transient and had disappeared 24 h after IP exposure (*8*). In our study IP-exposure to 50 mg BPA/kg bw did not significantly increase luc activity in the fetuses. It cannot be excluded that this was related to the higher dosage in our study, as the response in the study of Lemmen already was somewhat lower for the 10 than for the 1 mg/kg bw group after 8 h. Oral exposure did not result in luc-induction in the fetuses for any of the compounds. Interestingly, oral exposure to 1 mg EE/kg bw resulted in resorption of all fetuses. These EE dosages would be equivalent to about 2000 times the intake of EE via oral contraceptives which is about 0.5 µg/kg bw/day for a 60 kg female (*20*). Interestingly, resorptions did not occur in the E_2 or EP-dosed group exposed to a dose that represented a similar theoretical calculated total estrogenic potency based on *in vitro* EEF values (Table 1). The ethinyl group present in EE probably makes this estrogen more active *in vivo* compared to E_2 and EP. The relatively higher *in vivo* than *in vitro* estrogenic potency of EE is in accordance with results obtained previously, since with transgenic ERE-Luc zebrafish the EEF of EE increased from 1.2 *in vitro* to 100 in vivo (*21*). The fact that this increased relative *in vivo* potency is not observed for E_2 and EP but only for EE might be due to the greater persistence of EE against *in vivo* biotransformation. E_2 and EE induced the RLU/mg protein in almost all measured tissues and upon IP exposure these estrogens did cross the placenta as can be concluded from the luc induction in the fetuses (Table 3). Toxicokinetic studies in monkeys revealed that upon *iv* exposure to E_2 small amounts of estrogenic metabolites reached the fetal plasma (*22*).

Table 5: *In vivo* data overview of original research performed on suspected estrogenic active compounds.

Compound	Dosing & route	Species	Effect during exposure	Reference
E_2	1 mg/kg bw iv	Rhesus monkeys	Rapid metabolism to estrone, only 2% of E2 reached the fetus of the monkeys.	(*22*)
BPA	1 mg/kg bw ip	Heterozygous ER-luc mice	Significantly induced luc activity after 8 hours, however, after 24 hours exposure to 10 mg/kg bw, no significant effect was seen.	(*7,8*)
BPA	200 mg/kg bw sc and po	Ovariectomized prepubertal Long-Evans rats	Increased uterine wet weight in prepubertal rats in the uterotrophic assay, after both oral and sc exposure for three days from day 21-23 once daily.	(*41*)
BPA	50 mg/kg bw po	Pregnant Alderley Park rats	Increase in the age of vaginal opening after maternal exposure from GD6-21 was shown in the female offspring.	(*42*)
BPA	500 mg/kg bw, dietary exposure	Sprague-Dawley rats	No effects were seen on the uterus weight, however the amount of implantations and total pups/litter were affected after three generations of dietary exposure.	(*43*)
BPA	20 µg/kg bw sc	ICR (CD-1) mice	Offspring showed induction of early vaginal opening after maternal sc exposure from GD11-17. At 2 µg/kg bw BPA, however, reproductive functioning after mating with non-exposed males was not affected.	(*44*)
BPA	300 mg/kg bw sc and po	AP mice	Estrogenic effects in the uterotrophic assay in immature mice, were not dose-related present when exposed up to three consecutive days either sc or po.	(*45*)
BPA	3000 ppm in the diet (approximately 150 mg//kg bw/day).	Sprague-Dawley rats	After exposure maternal dietary exposure from GD15 to PND10, no significant effects were found on vaginal opening and estrous cyclicity.	(*46*)
BPA	25 - 250 ng BPA/kg bw via a maternal, osmotic pump	CD-1 mice	Increase in terminal ducts, end buds and alveolar buds (indicators for the development of breast tumors) after exposure from GD9 to the remainder of pregnancy.	(*47*)
NP	10 mg/kg bw sc	Immature CD-1 mice	Uterine wet weights were increased after sc exposure for three days.	(*48*)
NP	650 ppm (approximately 350 mg/kg) in the diet	Sprague-Dawley rats	Clear effects were seen on the relative uterus weight at PND 21 in the first generation, relative ovary weights in the second generation and in the third generation the day of vaginal opening was earlier after three generations of dietary exposure.	(*49*)
NP	3000 ppm in the diet (approximately 150 mg//kg bw/day).	Sprague-Dawley rats	After exposure maternal dietary exposure from GD15 to PND10, no significant effects were found on vaginal opening and estrous cyclicity.	(*46*)

Compound	Dosing & route	Species	Effect during exposure	Reference
NP	200 mg/kg bw sc and po	Ovariectomized prepubertal Long-Evans rats	Increased uterine wet weight in prepubertal rats in the uterotrophic assay, after oral exposure significant from 50 mg/kg and from 100 mg/kg after sc exposure for three days from day 21-23 once daily.	(*41*)
NP	50 mg/kg bw po	Sprague-Dawley rats	After exposure on GD 20, within 2-3 hours, 30-40% of the maternal NP concentration was measured in fetuses.	(*50*)
NP	2000 ppm in the diet	Sprague-Dawley rats	No estrogenic effects were present after exposure to NP in the diet up to three generations.	(*51*)
DEHP	2000 mg/kg bw po	Immature Ovariectomized Spague-Dawley rats	Affected the uterine weight after oral exposure for 4 days starting approximately 25 days of age, these effects could not be repeated with heavier rats.	(*52*)
DEHP	375 mg/kg bw po	Male offspring from Sprague-Dawley rats	After maternal oral exposure from GD3 to PND21 a general decline in prostate weight and other major reproductive defects were shown. Might be due to general toxicity then endocrine effects.	(*26*)
DIHP	2000 mg/kg bw po	Ovariectomized Spague-Dawley rats	Affected the uterine weight after oral exposure for 4 days starting approximately 25 days of age, these effects could not be repeated with heavier rats.	(*52*)
DEHA	800 mg/kg bw po	Mol:WIST rats	After oral exposure from GD7 to PND17 no effects on relevant organ weights affecting the endocrine system or the reproductive system were observed.	(*35*)

It cannot be excluded that the luc induction in the fetuses also was induced by estrogenic metabolites. It is of interest to know that, in spite of the blood-brain barrier, significant luc induction was observed in the female brain after oral but not after IP exposure to EE and E_2. The EP-induced luc-activity was not significantly different from control values. In the male mice exposed IP or orally to 1 mg EP/kg bw, luc activity in the brain was also significantly induced after 8 or 14 hours respectively (*9*). Probably after chronic oral exposure, more estrogens are able to pass the blood barrier than after short term IP-peak exposure as has been shown in rats (*23*).

In the present study IP-exposure to all three known estrogenic compounds (E_2, EE and EP) induced higher luc activities (RLU/mg protein) in fetuses than oral dosage. This is remarkable taking into account that the total IP dosage was 8 times lower than the total oral dosage. This higher luc induction by an 8 times lower dose might be explained by the rapid uptake of compounds in the blood after IP dosage compared to oral dosing, leading to higher maximal plasma values and thus higher concentrations in the placenta inducing the luc activity to a higher extent (*18*). This in contrast to the luc inductions in the liver that were higher after oral compared to IP exposure to the hormones, a phenomenon also observed in the male luc mice exposed to estrogens (*9*). This might be due to the fact that after oral uptake all estrogens are transported to the liver before entering the blood stream, which may result in relatively high liver exposure, compared to IP dosing.

In the present study both oral and IP-exposure to the food associated estrogenic compounds: BPA, NP, DEHA, DEHP, DIHP, DDE and quercetin were not able to induce luc activity in the examined tissues, including placenta and fetuses (Table 4). However, after oral exposure to NP, BPA and DIHP the luc activity in the placenta was lowered significantly. Reports on the reproductive and developmental effects of the *in vitro* estrogenic compounds also tested in this study are not conclusive. The most relevant studies concerning non-toxic exposures are summed in Table 5 and briefly discussed below.

In vivo estrogenic effects reported for BPA include increased uterine wet weight, and increased expression of ERα. However several other studies reported no in vivo effects upon BPA exposure (Table 5). In the present study, exposure to 50 mg BPA/kg bw in pregnant ER-luc mice did not result in a significantly increased luc activity in tissues of the mother or the fetuses. BPA even reduced the luc activity in the placenta (Table 4), which might explain the lack of effects in the fetuses. Possibly the placenta blocks or metabolizes the BPA thus protecting the fetuses. Also the extensive metabolism of BPA *in vivo* by adult mother mice (*24*) could explain the low luc induction in the fetuses and other tissues.

NP only slightly, but significant, reduced the luc activity in the placenta and fetuses upon IP exposure in ER-luc mice (Table 4). Estrogenic effects known from literature range from increased uterine wet weights to earlier age of vaginal opening in several species, although effects were not always consistent (Table 5). In general multiple generations are necessary to establish estrogenic effects of NP (Table 5), possibly explaining the lack of estrogenicity seen in the present study. This much lower *in vivo* than *in vitro* estrogenicity of NP has been shown before in ER-Luc transgenic zebrafish and was attributed to its *in vivo* biodegradability (*21*).

Both phthalates tested, DEHP and DIHP, did not induce luc activity in any of the measured tissues, which is in accordance with their very low estrogenic potencies (Table 1) and the very high dosages needed to induce effects *in vivo*. Even the most effective phthalate, DEHP, has been shown to induce estrogenic effects at oral dosages much higher than those of the present study (Table 5). As reviewed by Fisher (*25*), phthalates reduce testosterone levels during critical periods of development, but do not have an inducing effect on ER-luc activity, as shown in the present study as well. Based on the absence of effects from 100 mg/kg bw/day in the present study and assuming that the exposure of dialysis patients represents the maximum human exposure to DEHP, which is 12 g/year (*26*), equivalent to intravenous exposure for a 60 kg person of 0.55 mg/kg bw/day, no estrogenic effects from DEHP are expected in humans.

In our study DIHP significantly reduced the luc activity in the placenta upon both exposure routes (Table 4). As DIHP has been reported to be anti-estrogenic via the ERβ in the presence of E_2 (*27*) it is to be expected that especially in tissues with a high estrogenic background activity like the placenta (Table 3) the anti-estrogenicity can be detected. Phthalates also have been reported to suppress aromatase (*26,28*), which has an important function to transform testosterone into estradiol (*29*). Inhibition of aromatase might therefore also present a mechanism underlying the reduced luc-activity upon DIHP exposure.

Interestingly, all three compounds that reduced the luc activity in the placenta, DIHP, NP and BPA, are known to be anti-androgenic (*26,28,30,31*). Besides the possibility of being anti-androgenic in nature, the mechanism underlying the reduction of luc activity remains to be

elucidated. The effect could be caused by a reduction in the aromatase activity, an activity which is vital for normal human fetal development (*32*). Both aromatase, and 17β-hydroxy steroid dehydrogenase (17β-HSD) are upregulated during normal pregnancy in rats, and both are important for local estradiol synthesis (*33*). Therefore interference of endocrine disrupting compounds with aromatase gene regulation (*34*) and aromatase activity could theoretically result in lower estrogenic activity and thus be a mechanism for secondary effects.

Exposure to 100 mg DEHA/kg bw/day did not induce the luc activity in any tissue which is in line with previous studies in rats (Table 5). The only exception is the placenta in which luc activity was induced 1.2-fold after oral exposure only. IP-exposure to DEHA significantly reduced the luc activity in the placenta (Table 4), this difference between oral and IP exposure was not expected, and as DEHA is not known to be anti-androgenic, effects on a reduction of luc activity were also not expected (*35*).

p,p'-DDE did not affect luc activity in any of the organs or fetuses in the present study (Table 4), which might be due to its extreme lipophylicity. Due to storage of *p,p'*-DDE in the fat depots (*36*), *p,p'*-DDE will only be gradually released into the systemic circulation and effects are therefore expected only upon long term exposure (*21*).

Quercetin, which was estrogenic *in vitro* (*3*) and slightly estrogenic *in vivo* in ER-luc male mice (*9*), did not induce nor reduce the luc activity in any of the measured tissues in pregnant ER-luc mice (Table 4). The absence of effects in the female mice compared to the very slight effects in the male mice could be due to the already much higher background estrogenic activity in the female mice.

All together the present study clearly shows that the tested food-associated compounds known to be estrogenic *in vitro*, are not inducing luc activity in fetuses of orally exposed pregnant ER-luc mice during organogenesis, the crucial period of development. Alpha-fetoprotein in rodents is known to bind estrogens, thereby lowering the bioavailability to the fetus (*37*) and protecting the fetus from exposure to estrogens (*38*). The more artificial worst-case IP-exposure did significantly reduce the luc activity in the placenta, probably because this route of exposure results in high peak exposures that may temporarily exceed the binding capacity of alpha-fetoprotein, more than in the case of oral exposure resulting in more chronic lower concentrations.

The present results indicate that, at the current levels of exposure to food associated endocrine disrupting pseudoestrogens, estrogenic effects on the fetus are not to be expected. The revealed significant reduction in the placental luc activity, however, should be further studied for its significance for normal fetal development and relevance for the human situation.

Abbreviations:

DDE, 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethylene; EEF, Estradiol Equivalence Factor; EEQ, Estradiol Equivalents; ER, Estrogen Receptor; ER-luc, Estrogen Receptor reporter gene (luciferase); GD, Gestation Day; IF, Induction Factor; IP, Intraperitoneal; PND, Postnatal Day; RLU, relative light units.

Key words:

estrogen receptor, fetus, placental transfer, reporter gene mice, endocrine disruption, food packaging, plasticizer, pseudo-estrogen.

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Chapter 5:

Tissue distribution and fate of the estrogenic compounds estradiol, nonylphenol and p,p'-dichlorodiphenyldichloroethylene in pregnant C57black mice.

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5

Abstract

The tissue distribution and fate of estradiol and two pseudo-estrogens was studied in pregnant C57black/6J mice after oral exposure during early or late gestation with special emphasis on the amount recovered in the placenta and fetuses. The compounds were dosed as ^{14}C-radioactively labelled compounds ensuring a radioactive dose of 23 µCi/kg bw/day. Doses were 0.1 mg estradiol (E_2)/kg bw/day, used as a reference compound, 10 mg nonylphenol (NP)/kg bw/day or 1 mg *p,p*'-dichlorodiphenyldichloroethylene (*p,p*'-DDE)/kg bw/day. The compounds were fed daily in 0.5 g custard, either during gestation day (GD) 1-8 or GD9-16. Radioactivity was determined in tissues, carcasses, fetuses and excrements. Average total recovery was 41% for E_2, 16% for NP and 54% for *p,p*'-DDE, with the recovery inside the mice amounting to less than 0.2% of the total oral dose for E_2, 0.4% for NP, and 13.8% for *p,p*'-DDE. Most of the compounds was found in faeces and a lower amount in urine. The relative distribution of the compounds in the body did not differ between the exposure periods GD1-8 and GD9-16, but markedly differed between the 3 compounds. On average E_2 is recovered mainly in the liver at 23.5 ng/g liver (0.16% of the recovered compound) as was NP 2.2 µg/g liver (0.16%). The presence of 8.7 µg NP/g kidney (0.08%) shows that the kidneys also are a major route of NP-excretion. DDE was mainly present at levels of 14.3 µg/g in fat (0.82%) and 2.5 µg/g liver (1.9%).
The estrogen E_2 did not reach the fetus at a level above the DL, NP and *p,p*'-DDE did reach the fetus. Levels of E_2 and NP detected in placenta were relatively high, when compared to those in the fetuses, which might be due to prevention of transport to the fetuses by placental binding proteins. The results of the present paper reveal the existence of a variable level of the placental barrier function for some but not all (pseudo)estrogens, preventing in some but not all cases high systemic levels of circulating (pseudo)estrogens to actually reach the developing fetus. This indicates that without adequate *in vivo* validation, results from *in vitro* bioassays revealing estrogenic activity of (pseudo)estrogens are not enough to estimate the risk for fetuses.

5

Introduction

Pseudo-estrogens can interfere with normal estrogen homeostasis and interact with the estrogen receptors (ERs) thereby possibly impairing normal sexual development and fertility (*1*) and possibly increasing the risk on hormone-dependent cancers (*2*). For example the estrogen-responsive mammary gland is developed during embryonic life, so *in utero* exposure to estrogens could have implications for the risk of breast cancer later in life (*3-5*). Also epidemiological studies suggest that human fetuses exposed to elevated levels of estrogens or androgens *in utero* are at a relatively high risk of developing hormone-dependent cancers later in life (*6,7*).

Several estrogenic compounds have been detected in human fetuses and fetuses of exposed pregnant experimental animals. Bisphenol A was measured in human fetal plasma at levels of 2.3 ng/mL, which were close to maternal blood levels (3.1 ng/mL) (*8*), and in follicular fluid at 1-2 ng/mL (*9*). After oral exposure via gavage of pregnant Wistar rats from gestation day (GD)3 to GD19 to a total dosage of 1 µg/kg bw radioactively labelled 4-n-nonylphenol (4-n-NP), no significant amount was recovered in the fetus (*10*). After oral exposure via gavage of pregnant Sprague Dawley (SD) rats to 10 mg *p,p'*-dichloro-2,2-bis(4-chlorophenyl)ethylene (*p,p'*-DDE)/kg bw/day from GD14-18 in fetuses an amount of 0.059 µg *p,p'*-DDE /g fetus was recovered at GD19, placental concentrations however were almost 3 times higher namely 0.153 µg *p,p'*-DDE /g (*11*). Earlier studies with dietary exposure of pregnant SD rats to 200 mg *p,p'*-DDT/kg feed (being equal to approximately 12.5 *p,p'*-DDT mg/kg bw/day assuming a body weight of 400 gram and a feed intake of 25 gram/day for a pregnant rat), resulted in levels in the fetuses of 2 µg DDE/g kidney in neonates which were about 3 times lower than levels detected in the dam (*12*). These authors also noted that after *iv* exposure of the dam, the levels of DDE in the maternal tissues were significantly higher in placenta than in uterus or plasma.

Figure 1: Mouse eating its 0.5 ml of vanilla custard mixed with test compound from a small stainless steel bucket. An animal-friendly way of quantitative oral exposure.

In our previous studies with pregnant transgenic ER-reporter gene mice only IP but not oral exposure to estrogenic food-associated compounds affected luciferase activity in fetuses. After oral exposure for 8 days from GD8 to GD15 significant effects were observed on the placentas only, after exposure to 50 mg/kg bw nonylphenol, 50 mg/kg bw bisphenol A, 100 mg/kg di-isoheptyl phthalate (DIHP), and 100 mg/kg bw di-(2-ethylhexyl) adipate (DEHA) (*13*). This suggests that these compounds do not reach the fetus but may accumulate in placental tissue. The goal of the present study was to determine to what extent two

different radioactive labelled pseudo-estrogens (*p,p'*-DDE and 4-NP) and the natural estrogen estradiol (E_2) can reach the fetuses in orally exposed pregnant C57black mice. The C57black mice are of the same strain as the genetically modified ER-Luc mice in which we studied the estrogenic responses in fetuses and placenta, so the toxicokinetics of the compounds in the two experiments are expected to be the same. Two exposure periods were selected, namely early (GD1-8) and late (GD9-16) exposure, encompassing, respectively, the start and major part of organogenesis and the latter part of pregnancy which includes sexual differentiation (*5*). The tissue distribution and fate of the estrogenic compounds is studied and the levels reached in the fetuses and placenta were compared with each other.

Materials and methods

Animals

The animal experiment was approved by the Animal ethics committee of Wageningen University. Thirty-seven female proven breeder C57black/6J and 18 male mice were obtained from Charles Rivers (The Netherlands). The female mice were acclimatized for three weeks in rooms separate from male mice to synchronize the oestrous cycles for the maximal "Whitten" effect (*14*). All mice were fed a phyto-estrogen free diet (AM-II diet, Hope Farms, The Netherlands) *ad libitum*.

After the acclimatization period the mice were transferred to Macrolon cages with two small compartments in which a male and a female were kept separated for two days after which the two compartments were united and the mice were allowed to mate. Twelve hours later, the females were checked for a vaginal plug and this was repeated twice a day for four consecutive days. The moment a vaginal plug was found was designated gestation day 0 (GD0).

Experimental set-up

The pregnant mice were orally exposed to a mixture of radioactively labelled and non-radioactive compounds. The total dose of radioactivity was 23 uCi/kg bw/day in each group, which is high enough to detect the most important routes of the compounds. However, because of the high specific radioactivity of the compounds (given below) the absolute amount of compound dosed would be far too low to expect toxicokinetics comparable to that of more common higher dosages reported for other studies. Therefore non-radioactive compound was added to reach higher, still non-toxic, dosages. The total exposure levels amounted to 0.1 mg/kg bw/day for estradiol (E_2; CAS 50-28-2), 10 mg/kg bw/day for nonylphenol (NP; CAS 104-40-5) and 1 mg/kg bw/day for *p,p'*-dichloro-2,2-bis(4-chlorophenyl)ethylene (*p,p'*-DDE; CAS 72-55-9).

The exposure of E_2 was based on Lemmen *et al.* (*15*), showing 1 mg/kg/day of EP being estrogenic. As E_2 is expected to be more or less similar in potency to EP, and as the present study was conducted to study the fate and distribution of E_2 and not estrogenicity, a dosage 10 times lower was chosen. The lowest dosage used in our previous study in male ER-Luc mice was used to select the dosage for NP (*16*). The dosage of *p,p'*-DDE was chosen between E_2 and NP. E_2 (>98%) and *p,p'*-DDE (>99%) were obtained from Sigma (The Netherlands), NP (~85%; technical mixture) was obtained from Fluka (Germany). The radioactively labelled

4-^{14}C E_2 had a specific activity of 55 mCi/mmol, ring-^{14}C *p'p'*-DDE a specific activity of 13 mCi/mmol and ring-^{14}C NP a specific activity of 52 mCi/mmol. All radioactively labelled compounds were obtained from American Radiolabelled Chemicals (MO, USA). All compounds were dissolved and diluted in ethanol (>99,9%) from Merck (Germany) and 6 µl/gram bw of the stocks were daily freshly mixed in 0.5 ml vanilla custard of organic origin (Demeter, The Netherlands). Dosing via vanilla custard avoided discomfort due to oral gavage and four days before the actual exposure, the mice were accustomed to eat vanilla custard from a small stainless steel beaker, one for each animal (Figure 1). During the exposure period, mice were weighed daily and housed on filter paper to absorb the radioactivity present in the urine. Exposure took place either from GD1-8 or from GD9-16 after which, on GD9 or on GD17, blood was collected via orbita extraction under isoflurane anesthesia (Abbott Laboratories Ltd., Berkshire, England). Two droplets heparine (5000 IU/ml, Leo pharmaceutical products BV, Weesp, The Netherlands) were added to 1.5 ml Eppendorf vials (Eppendorf, Hamburg, Germany) to avoid clotting of the blood. Plasma was collected after centrifugation at 10000 g for 15 min at 4°C. Subsequently, mice were cervically dislocated and several maternal tissues were collected (see "radioactivity measurement") and stored at -20ºC until further analysis. On GD9 placentas and fetuses could only be collected together as one sample because of their small seize. On GD17 fetuses and placentas were collected separately. Fetuses were decapitated as soon as they were separated from the placenta.

Radioactivity measurement

From all tissues, two samples of at least 25 mg were weighed and dissolved in 1 ml Soluene-350 (PerkinElmer, Boston, MA) and incubated during four hours at 50ºC. To avoid quenching from the tissue colour all solutions were discoloured by adding a specific amount of a 30% H_2O_2 solution (Sigma-Aldrich, Steinheim, Germany) at room temperature. To the adrenals, plasma, brain, abdominal fat, placenta plus fetuses (GD1-8), gall bladder, kidneys, liver, thymus and uterus (GD9-16) 300 µl H_2O_2 was added. To the fetuses (GD9-16) 400 µl H_2O_2, was added, to the blood pellet and placenta 600 µl H_2O_2, to the gut without faeces 800 µl H_2O_2, to heart and spleen 1000 µl H_2O_2. Faeces were grinded with a mortar and to 25 mg 1 ml Soluene-350 and subsequently 0.5 ml isopropanol (99%, Sigma-Aldrich, Steinheim, Germany) were added. After two hours incubation at 50ºC 2 ml H_2O_2 solution was added. Fetuses from GD9-16 were first dissolved in 1 ml 1.5 M KOH, 20% EtOH, then homogenized with the ultra thurrax, and 0.3 ml of this sample was used for further processing as described above. Of the filter paper (used as dissecting underground) 1 gram was extracted for two days with 25 ml di-isopropyl ether (99%, Sigma-Aldrich, Steinheim, Germany), 0.2 ml of this extract was used for further processing starting with Soluene-350 addition as described above. Carcasses of the mother mice were dissolved in 100 ml 1.5 M KOH, 20% EtOH for two days and homogenized with the Ultra Thurrax, 0.3 ml from this homogenate was discoloured with 1800 µl H_2O_2 solution.

To all dissolved and discoloured samples 18 ml scintillation fluid, (Hionic Fluor, PerkinElmer, Boston, MA), was added in 20 ml plastic scintillation vials (Greiner, Alphen a/d Rijn, The Netherlands), mixed and stored for at least 12 hours at room temperature prior to measurement to avoid quenching by the tissue colour. For two independently prepared samples, the disintegrations per minute (DPM) were counted during five minutes in a Packard 1600 liquid

Table 1: Average levels of the compounds recovered from tissues of mice exposed during eight days either during gestation day (GD)1-8 or GD 9-16. The amounts are expressed as ng compound/g tissue, ng compound/tissue and % of the total recovered dosage. Detection limit

		DL	E_2	
Exposure period:			**GD1-8**	**GD9-16**
Daily dosage	ng/gram bw/day		100	100
Total dosage over 8 days	ng per mouse		19 595±1011	20 425±163
Totally recovered	ng		8 769±2 762	7 531±69
	% of dosage		45.2±16.4	36.9±3.0
Recovered in the animal[4]	ng		42.2±8.9	41.1±8.6
	% of dosage		0.2±0.05	0.2±0.04
Internal tissues				
Adrenal (L+R)	ng/g tissue	2.4	*0.8±0.3*	*-2.1±0.7*
	ng/tissue	0.01	*0.009±0.009*	*-0.01±0.002*
	%		*0.00004 ±0.00001*	*-0.00007 ±0.00001*
Blood pellet	ng/g tissue	1.6	*0.4±0.2*	*1.2±0.1*
	ng/tissue	1	*0.3±0.3*	*0.5±0*
	%		*0.002±0.001*	*0.003±0.002*
Blood plasma	ng/g tissue	3	*0.7±1.0*	*0.4±0.4*
	ng/tissue	1.6	*0.4±0.6*	*0.2±0.1*
	%		*0.002±0.003*	*0.0008 ±0.0007*
Brain	ng/g tissue	0.7	3.3±5.1	*-0.1±0*
	ng/tissue	0.3	1.5±2.3	*-0.1±0*
	%		0.01±0.01	*-0.0003 ±0.00003*
Abdominal Fat[1]	ng/g tissue	0.6	*0.6±0.1*	*0.5±0.2*
Heart	ng/g tissue	1.1	*0.4±0.5*	*0.8±0.1*
	ng/tissue	0.1	*0.05±0.08*	*0.1±0.01*
	%		*0.0003±0.0004*	*0.0005 ±0.00005*
Lung	ng/g tissue	0.8	*0.8±0.4*	*0.3±0.3*
	ng/tissue	0.1	*0.1±0.1*	*0.04±0.04*
	%		*0.0006±0.0003*	*0.0002 ±0.0002*
Spleen	ng/g tissue	1.8	*0.4±0.1*	*0.9±0.1*
	ng/tissue	0.2	*0.05±0.02*	*0.07±0.009*
	%		*0.0003±0.0001*	*0.0004 ±0.00005*
Thymus	ng/g tissue	0.8	*0.7±0.6*	*-0.3±0.6*
	ng/tissue	0.03	*0.03±0.03*	*-0.01±0.02*
	%		*0.0002±0.0001*	*-0.00006 ±0.0001*
Average internal levels	Average ng/g tissue		0.9±0.9	0.2±0.9

(DL) is set as 2xSTDEV of the tissue- and compound-specific controls. Levels below the statistically defined DL, taken as two times the standard deviation obtained for the samples from the control mice, are printed in *italic*.

DL	NP		DL	DDE	
	GD1-8	**GD9-16**		**GD1-8**	**GD9-16**
	10000	10000		1000	1000
	1821 500±14 849	2280 400±257 696		197 600±4 243	230 500±990
	324 486±8 9867	341 187±83 317		99 832±8 874	133 879±37 010
	17.8±5.1	14.8±2.6		50.6±5.6	58.0±16.0
	6968±953	9085±1214		36 115±7 820	42 158±9 368
	0.4±0.05	0.4±0.05		18.3±4.0	18.3±4.1
242	510±66	895±232	24.2	3688±1013	6523±1581
1	4.2±1.6	7.4±1.2	0.1	28.8±1.7	68±23
	0.0002±0.00009	0.0003±0.00005		0.01±0.001	0.03±0.01
159	*32±11*	*79±70*	15.9	227±187	71.3±35.9
97.5	*17.3±6.1*	*52±53*	9.8	155±132	53±33
	0.001±0.0003	*0.002±0.002*		0.08±0.07	0.02±0.01
295	119±25	69±45	29.5	226±320	381±58.9
164	67.4±0.5	46±29	16.4	154±218	253±71
	0.004±0.000003	0.002±0.002		0.08±0.11	0.11±0.03
66.6	725±291	155±357	6.7	48.1±26.9	134±33
28.9	316±132	64±150	2.9	22±13	58.5±15.8
	0.02±0.01	0.003±0.007		0.01±0.007	0.03±0.007
64.5	174±19	121±41	6.5	14 811±4 093	13 723±2 230
109	1043±72.3	1287±133	10.9	349±219	289±6.6
12.9	129±12.6	169±22	1.3	50±34	37.4±1.0
	0.007±0.0006	0.007±0.001		0.03±0.02	0.02±0.0005
78.7	294±2.0	296±67	7.9	1213±312	1853±315
11.6	31.7±6.2	44.7±9.6	1.2	190±26	285±35
	0.002±0.0004	0.002±0.0006		0.10±0.02	0.12±0.02
176	320±62	469±202	17.6	254±136	306±27
15.5	36±6.7	45±12.5	1.6	30±15	27±3
	0.002±0.0004	0.002±0.005		0.02±0.01	0.01±0.001
75.4	452±35	488±112	7.5	1232±1204	3806±93
2.7	17.5±5.8	14.9±7.8	0.3	53±62	115±6.6
	0.001±0.0003	0.0007±0.0005		0.03±0.03	0.05±0.003
	408±320	429±418		2448±4774	3009±4577

Tissues involved in elimination & outside body				
		DL	E_2	
Exposure period:			**GD1-8**	**GD9-16**
Liver	ng/g tissue	0.7	25.2±0.4	21.8±1.6
	ng/tissue	0.9	32.2±2.5	32.2±0.7
	%		0.16±0.004	0.16±0.004
Bile	ng/g tissue	1.2	13.9±3.5	10.2±1.2
	ng/tissue	0	0.2±0.1	0.2±0.2
	%		0.001±0.0007	0.001±0.0008
Kidney (L+R)	ng/g tissue	2	2.6±1.1	2.6±0.03
	ng/tissue	0.3	0.5±0.2	0.5±0.03
	%		0.002±0.0009	0.002±0.0001
Carcass	ng/g tissue	4	*0.4±0.06*	*0.3±0.1*
	ng/tissue	69	*6.8±0.7*	*6.3±2.6*
	%		*0.03±0.01*	*0.03±0.01*
Gut	ng/g tissue	1.6	4.3±0.7	3.7±1.3
(incl. contents)	ng total	4.5	11.0±2.1	9.3±3.1
	%		0.06±0.008	0.05±0.02
Faeces	ng/g faeces	37.5	1 166±423	1 287±120
	ng total	230	8714±2765	7479±678
	%		44.9±16.4	36.6±3.0
Filter paper	ng total	2.7	*0.8±1.3*	*0.7±0.4*
	%		*0.004±0.007*	*0.004±0.002*
Bedding	ng/g paper	0.008	*0.006±0.006*	*0.006±0.007*
	ng total	0.5	*0.5±0.4*	*0.3±0.4*
	%		*0.003±0.002*	*0.002±0.002*
Reproductive tissues and fetuses				
Uterus	ng/g tissue	0.8	*0.8±0.1*	1.4±0.3
	ng/tissue	0.1	0.2±0.1	0.6±0.6
	%		0.0009±0.0003	0.003±0.003
Fetus & Placenta	ng/g tissue	0.7	*0.1±0.6*	[3]
	ng/tissue	0.02	*0.005±0.01*	[3]
	%		*0.00002±0.00006*	
Fetus	ng/g tissue	0.7	nd[2]	*0.5±0*
	ng/tissue	0.3	nd[2]	*0.3±0.03*
	%			*0.001±0.0002*
Placenta	ng/g tissue	1.4	nd[2]	2.6±0.03
	ng/tissue	0.2	nd[2]	0.3±0.02
	%			0.002±0.0001

[1] Only a sample of the abdominal fat was taken, the total body fat was not determined
[2] nd is not determined because fetuses and placenta were too small to collect separately
[3] Fetuses and placenta could be measured separately
[4] All compound recovered excluding the faeces, bile, bedding, filter paper and gut with content

DL	NP		DL	DDE	
	GD1-8	GD9-16		GD1-8	GD9-16
67.9	2284±566	2224±467	6.8	3339±679	1737±210
88.7	2962±926	3953±525	8.9	4877±139	3037±557
	0.16±0.05	0.17±0.03		2.47±0.12	1.32±0.25
118	*-50±292*	335±313	11.9	420±594	3107±1545
1.2	*1.1±2.7*	3.6±3.1	0.1	1.6±2.2	44±52
	0.00006±0.0002	0.0002±0.0002		0.0008±0.001	0.02±0.02
197.7	7 879±2135	9 601±1876	19.8	1 615±65.5	1 652±31
30.7	1 358±459	1 776±342	3.1	301±15.8	297±21
	0.07±0.02	0.08±0.02		0.15±0.004	0.13±0.01
401	*111±65.6*	*128±21.8*	40.1	1513±146	1766±138
69301	*1972±1166*	*2475±437*	693.1	28 421±3 019	35 413±1 301
	0.11±0.06	*0.11±0.02*		14.4±1.8	15.36±0.63
164	590±169	1176±216	16.4	847±97.5	474±32
447	1419±452	3960±1226	44.7	2553±674	1562±38
	0.08±0.02	0.17±0.04		1.29±0.31	0.68±0.01
3751	46 554±8 795	42 983±8 768	375	9 683±1135	12 707±5 955
23002	315 966±92 800	327 981±82 277	2300	59 861±3 929	89 301±40 586
	17.37±5.24	14±2.7		30.32±2.64	39±17
2712	*-35±48*	*35±46*	27	1 205±1555	677±543
	-0.002±0.003	*0.001±0.002*		0.62±0.80	0.29±0.24
0.8	2.6±1.7	1.8±1.3	0.08	0.6±0.4	0.4±0.02
47.9	150±81	107±81	4.8	42±26	22±1.5
	0.008±0.004	0.005±0.004		0.02±0.01	0.009±0.0006
76	375±62	481±82	7.6	2 595±1212	578±314
13.5	51±12	321±185	1.4	411±63	467±303
	0.003±0.0006	0.01±0.007		0.21±0.03	0.20±0.13
72	149±28	[3]	7.2	203±43	[3]
1.8	2.9±0.1	[3]	0.2	3.3±0.3	[3]
	0.0002±0.00001			0.002±0.0002	
70	nd[2]	168±125	6.9	nd[2]	477±448
32	nd[2]	54±30	3.2	nd[2]	85±22
		0.002±0.001			0.04±0.01
105	nd[2]	437±264	14.1	nd[2]	419±76
18	nd[2]	61±39	1.8	nd[2]	60±9.4
		0.003±0.002			0.03±0.004

scintillation counter (LSC, 1600 TR, Packard, Downer's grove, IL). The tissues of the control mice were processed in a similar way and measured to determine the background signals.

Data processing and statistical analysis
Based on the average reading (DPM) for the tissues from the control mice the detection limit (DL) was set at two times the standard deviation obtained for the samples from the control mice. Values higher than this DL were used for further calculations after correction for the value of the respective tissue from the control mice. Values lower than the statistically defined DL are presented in Table 1 in *italics*. Based on the ratio between the total radioactivity and the total amount of compound dosed to the animals, the overall recovery was calculated and the relative radioactivity recovered in each tissue was presented as the average amount of radioactivity per total tissue and per g tissue expressed as % of the total recovered dosage. When corrected for DPM values of the control mice, DPM values were further transformed with the knowledge that $2.22 \cdot 10^6$ dpm equals 1 µCurie. 23µCurie in this study equals either 0.1 mg/kg bw/day for E_2; 10 mg/kg bw/day for NP; or 1 mg/kg bw/day for *p,p*'-DDE, based upon these facts, final concentrations of E_2, NP or *p,p*'-DDE in the tissues were calculated. Differences between two groups of mice were tested for significance using a student's T-test with a *p*-value of 0.05 (SPSS 10.1.0 for Windows).

Results

The breeding success in the present study using synchronization of the oestrous cycles of the females was 15 pregnancies out of 37 C57black/6J female mice within a time-frame of four days. Within the habituation period of four days before the start of the exposure, all mice learned to appreciate the vanilla custard and finished it quickly without spoilage (Figure 1). During the whole exposure period all mice completely finished their 0.5 ml custard within ten minutes, showing this to be a very animal-friendly method for quantitative oral dosage administration.
Of the total amount of radioactivity given to the animals (23 µCi/kg mouse), an average of 41% was recovered for E_2, 16% for NP and 54% for *p,p*'-DDE (Table 1). The vast majority of the recovered compound was found in the faeces (40% of the orally administered dose for E_2, 16% for NP and 34% for *p,p*'-DDE) and to a lesser degree in the filter paper under the animals during autopsy (0.6% recovered for *p,p*'-DDE) (Table 1). In the internal tissues of the mother animal (adrenal, brain, carcass, fat, heart, lung, blood pellet, blood plasma, spleen and thymus) on average 0.05% of the totally recovered dosage was found for E_2, 0.13% for NP and 16% for *p,p*'-DDE (Table 1). This means that no more than 0.02% of the total given dose of E_2 and NP, and 5.4 % of *p,p*'-DDE was recovered in the specified internal tissues of the mother mice. The rest of the compounds was recovered in tissues involved in metabolism and excretion (liver, kidneys, bile) and in reproductive tissues (uterus, placenta) and to a smaller extent in the fetuses. The relative distribution of the compounds hardly differed between the two exposure periods, GD1-8 or GD9-16, but differed markedly between the 3 compounds (Table 1). *p,p*'-DDE was mainly found in fat and fatty tissues including the remaining carcass. E_2 was mainly present in liver and bile, and NP in liver and kidneys (Table 1).
Because of their limited size at GD1-8, the placenta and fetuses could only be separated

and analysed individually at GD17 (so after exposure from GD9-16). At this time point for E_2 levels recovered in the placenta were 5 times higher than the average levels detected in the fetuses (0.5 ng/g). In most tissues, and also in fetuses of E_2 exposed mice levels of E_2 remained below the statistically defined DL (Table 1). The levels for NP, and *p,p*'-DDE were above the DL for both the fetuses as well as the placenta. The NP levels in the placenta were 3 times higher than the average levels in the fetuses. The *p,p*'-DDE levels in placenta and fetus were comparable. Compared to maternal blood plasma levels the levels in the placenta were higher for all 3 compounds tested. This difference was significant for E_2 and NP but not for *p,p*'-DDE.

Discussion

In the present study pregnant C57 black/6J mice were orally exposed to a mixture of radioactively labelled and unlabelled estradiol (E_2), nonylphenol (NP) and *p,p'*-DDE in order to study the fate and distribution of these compounds during early (GD1-8) or late pregnancy (GD9-16). The mice were exposed orally via vanilla custard which they voluntarily ate completely and without spilling from steel cups, strongly improving the dosing method with respect to the level of possible discomfort compared to exposure via oral gavage. The experimental groups of pregnant mice were unexpectedly small as only 15 out of 37 mice got pregnant within the chosen time-frame of four days, even though proven breeders were used. Of the total dosages given only 16% (NP), 41 % (E_2) and 54% (*p,p*'-DDE) was recovered, most of this in faeces and urine. The relatively low recovery could possibly be partially explained by losses via faeces or urine remaining in the cages, via remaining radioactivity in the feed cups, or via excretion via exhaled air and/or evaporation.
Of the total E_2 dosage given only 0.2% was recovered in tissues of the body (so excluding the faeces, bile, bedding, filter paper and gut with content) (Table 1). This is 0.5% of the totally recovered dosage. Excretion is mainly via the faecal route, and levels in liver and bile are highest suggesting metabolism and conjugation in the liver followed by biliary excretion. Upon oral exposure to tritium-labelled (^{3}H-) E_2 much higher recoveries have been reported, up to 75% within 2 days (*17*). This can to a large degree be attributed to rapid exchange of ^{3}H with water, which was confirmed by the high amounts of tritiated water recovered in the mice especially after oral exposure (*17*). An important difference also is that in the Fullerton study urine and faecal samples were collected 4 times during the 48 hours, the first 2 times after intervals of 4 hours, whereas in the present experiment the urine (bedding) and faecal samples were collected after eight days. During this period the excreted E_2 and its metabolites, may have been further degraded under influence of e.g. bacteria, UV light, and evaporation from especially the urinary excrements in the bedding but also the faeces. This might, explain the relative small recovery in the current study. In our study no E_2 above the DL was detected in the blood, although it is to be expected that E_2 and its metabolites might be bound to albumin, sex hormone binding globulin or alpha-fetoprotein in rodents (*17,18*). After maternal exposure E_2 was able to reach the brain in levels above the limit of detection but not the fetuses (Table 1). Significant levels, however, were found in the placentas which were five fold higher compared to the levels in the fetuses which were just below the DL (Table 1). No studies were reported in which E_2 was detected in significant amounts in the

fetuses after oral exposure. Only after unrealistically high peak exposure, e.g. via a catheter directly in an interplacental artery in rhesus monkeys (*19*) is the placenta not able to prevent E_2 from entering the fetuses. The low levels of E_2 in fetuses observed in the present study are in accordance with our previous results obtained in ER-Luc female mice where oral exposure to E_2 during pregnancy did not induce luciferase expression in the fetuses (*13*).

Of the total NP dosage given, only an average of 16% was recovered, of which 0.4% in the internal organs of the animals, which is on average 2.3% of the total dosage given (Table 1). Most radioactivity was recovered in the faeces and the liver (Table 1). Zalko and colleagues orally exposed Wistar rats to 1 µg and 10 mg NP/kg bw and recovered 68 up to 79% of the given NP dosage, of which approximately 20% was recovered in the faeces and 50% in the urine (*10*). This greater total recovery compared to our 16% can be the result of their daily collecting urine and faeces, probably resulting in less loss of NP than in our approach collecting this after 8 days only. NP was shown to be degraded into especially relatively small volatile molecules (*10*) which can easily be externally degraded and evaporated. For us the distribution in the body, and especially the relative amounts recovered in fetuses and placenta, was the focus of the research, not the total recovery. The relatively high NP-levels found in the kidneys, up to four times higher than in the liver, and undetectable levels in the bile (Table 1) suggest that also in our mice excretion via the urine is important. Major excretion via the urine and faeces is also in accordance with a risk assessment report from the European Union, concluding that in rodents neither NP nor its metabolites are excreted via exhalation, but that on average 70% is excreted via the faeces, 20% via the urine and only 0.4% is retained in tissues (*20*). This latter value is in line with the results of the present study. Humans are expected to only excrete 1.5% of NP via the faeces and 10% via the urine, with the remainder being unaccounted for (*20*). In the present study NP was detected in the fetuses combined with placentas after maternal exposure at GD1-8, these levels were however not significantly different from control levels and just twice the DL. The NP detected in these combined fetal and placental samples was probably due to the presence of NP in the placenta as after administration of NP during GD9-16 the NP levels in the placenta were significantly, 2.6 times, higher compared to the levels in the fetuses (Table 1). The results are in accordance with the results previously reported, with orally NP-exposed pregnant ER-Luc mice (*13*), where no significant effects were observed in the fetuses, but NP significantly affected, i.e lowered luciferase activity in the placenta. This reduction was hypothesized to result from a possible NP mediated reduction of aromatase activity (*13*).

Of the *p,p*'-DDE dosed 54% was recovered, of which 18.3% in the body, about 33.7% of the totally given dose. The high total recovery of *p,p*'-DDE and its possible metabolites within the animals is due to the persistent nature of the molecules and their low volatility. Because of their high lipophilicity, recovery was mostly in fat and faeces, which has been shown before for humans as well (*21,22*). Not only in the abdominal fat, but also in relatively small organs such as the adrenals, embedded in fat, high levels of *p,p*'-DDE were found (Table 1). In contrast to the situation with E_2 and NP, *p,p*'-DDE will be hardly metabolized and the levels will accumulate in fat tissue over time (*21*). The mice contained 10 times higher levels of *p,p*'-DDE in their abdominal fat than in their liver, which is in accordance with data reported on DDT by Gingell and colleagues (*23*). In our study *p,p*'-DDE accumulated to the same degree in the fetus as in the placenta (GD9-16) and the

combination of the placenta plus fetus (GD1-8) (Table 1). In a study reported in the literature *p,p*'-DDE was recovered both in fetuses and placentas after two daily maternal oral dosages in the period GD14-18, with the fetal levels being 2-3-fold lower than the placental levels (*11*). The difference with our findings showing comparable *p,p*'-DDE levels in placenta and fetuses could be due to the 4 times longer exposure in our study allowing more time for *p,p*'-DDE accumulation in the fetuses.

In humans *p,p*'-DDE has been found in placenta's (levels up to 18 µg/kg) (*24*), amniotic fluid (up to 0.15 ng/ml) (*25*), and in the fetal cord (up to 1.9 µg/kg) (*26*). However, in the placentas and fetuses of pregnant ER-Luc mice *p,p*'-DDE did not affect luciferase activity (*13*), which could be due to the small amount of compound accumulating in the fetuses and placenta within 8 days and/or their relatively low estrogenic potency.

Overall the present study reveals a very low internal recovery of the orally administered E_2 (0.2%) and NP (0.4%) and a slightly higher internal recovery for *p,p*'-DDE (18%). There were no clear differences in fate and recoveries of the compounds dosed during early or late pregnancy but the fate of the compounds in the maternal body differed greatly. The fetus could be reached by NP and *p,p*'-DDE, but not by E_2 in levels above the DL for radioactivity. This might be due to the presence of binding proteins such as alpha-fetoprotein, which can bind E_2 and prevent it from reaching the fetus, but possibly does not as effectively bind NP and *p,p*'-DDE. The levels of E_2 in the placenta were 5-fold and significantly above the detection level and thus at least 5 fold higher than what might have reached the fetuses, while this difference was 2.6 times and not significant for NP and absent for *p,p*'-DDE. Although the presence of compounds in placenta and fetuses do not automatically imply adverse effects, it does raise concern about the possible consequences for humans. Fortunately actual human exposure levels are lower than the ones used in this study, since the daily intake for NP is estimated to be around 0.00125 mg/kg/day (*27*), and for *p,p*'-DDE intake levels are even lower at approximately 0.00057 mg/kg/day (*28*). The direct translation of *in utero* exposure in mice to *in utero* exposure in man cannot easily be made. For humans it has been shown that alpha-fetoprotein does not bind pseudoestrogens such as diethylstilbestrol (DES) and therefore does not provide adequate protection of the fetus (*29*). Furthermore, *p,p*'-DDE (*24-26*) and other suspected endocrine disrupting compounds (*30,31*) have been found in human fetuses and amniotic fluid and the effects of the combination of these compounds are not yet known. In addition, effects on the functioning of the placenta, as has been shown with the reduced luc-activity in the transgenic mice (*16*), can have consequences for the developing fetuses as well.

Nevertheless the results of the present paper reveal the existence of a variable level of the placental barrier function for some but not all (pseudo)estrogens, preventing in some but not all cases high systemic levels of circulating (pseudo)estrogens to actually reach the developing fetus. This indicates that without adequate *in vivo* validation, results from *in vitro* bioassays revealing estrogenic activity of (pseudo)estrogens can not be interpreted as to indicate a risk for fetuses.

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Keywords:

Nonylphenol, dichlorodiphenyldichloroethylene (*p,p*'-DDE), estradiol, C57black mice, fetus, endocrine disruption, estrogenic, pseudo-estrogen, placenta .

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Chapter 6:

Summary and concluding remarks

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6

The aim of the present thesis was to study the *in vivo* relevance of *in vitro* detected estrogenic effects of food associated compounds, with emphasis on prenatal exposure. The results obtained reveal that the extent at which *in vitro* estrogenic effects may result in *in vivo* estrogenicity may vary with the compound under study and should be evaluated on a case by case basis. Differences in absorption, distribution, metabolism and excretion may all underlie the reasons why *in vitro* estrogenic compounds may exert their estrogenicity *in vivo* to a varying extent. The results of the present study reveal that available *in vitro* reporter gene bioassays can be of help in elucidating the intrinsic estrogenic potency of chemicals for either the ERα or ERβ. *In vivo* reporter gene bioassays can reveal whether the *in vitro* observed estrogenicity will actually result in tissue-specific responses *in vivo*. It was also demonstrated that these bioassays may be of help in defining safer alternatives for plasticizers currently used in food packaging.

In Chapter 2 of the present thesis the estrogenic potency of 21 food packaging-associated compounds was studied in ERα or ERβ transfected U2-OS (human osteoblasts devoid of endogenous estrogen receptors) cell lines. Six plasticizers and three anti-oxidants were slightly estrogenic in the ERα cells. BPA, NP, one plasticizer (tris (2-ethylhexyl) trimellitate (TEHTM)) and two anti-oxidants (propyl gallate and butylated hydroxy anisole) were estrogenic both in ERα and ERβ cells. Compared to E_2, these compounds appeared to be relatively more estrogenic in the ERβ cells than in the ERα cells. The differences in ligand binding domains of the ERα and the ERβ (*1, 2, 3*) are one explanation for differences observed regarding ERα or ERβ activation by different compounds in Chapter 2. The three sorbitol-based plasticizers tested did neither activate ERα nor ERβ and may be good replacements of existing plasticizers. The food-associated estrogenic compounds, may contribute to the total pool of estrogenic compounds humans are exposed to. The estrogenic potencies of our compounds, together with the suggested beneficial effect of ERβ mediated responses and adverse effects of ERα mediated responses (*4*), support the importance of detecting characteristics for ERα and ERβ responses separately in independent models. Currently only *in vitro* models allow quantification of differences in the relative potency of chemicals towards these two estrogen receptors. However, chemicals and thus also estrogenic compounds may show different kinetics, i.e. absorption, distribution, metabolism and excretion characteristics, which will only become apparent in *in vivo* models. Therefore, in Chapter 3 the seven food-associated compounds, shown to be estrogenic in our *in vitro* studies in Chapter 2, were studied for their *in vivo* estrogenic effects in male mice with an ER-reporter gene (luciferase) construct inserted.

Male ER-Luc mice were dosed either IP or orally for 8 or 14 hours respectively and the luciferase (luc) induction was determined in different tissues. 17β-Estradiol 3,17-dipropionate (EP) was used as a positive control at 0.3 and 1 mg/kg bw, DMSO as a solvent control. The food-associated estrogenic compounds tested at non-toxic doses were: BPA and NP (both at 10 and 50 mg/kg bw), *p,p'*-DDE; at 5 and 25 mg/kg bw, quercetin (at 1.66 and 16.6 mg/kg bw), DIHP, DEHP and DEHA all at 30 and 100 mg/kg bw. In general IP dosing resulted in higher luc-inductions than oral dosing. EP induced luc-activity in the liver in a statistically significant dose-related way with the highest induction of all compounds tested, which was

20,000 times higher than the induction by the DMSO-control. NP, DDE, DEHA and DIHP did not induce luc-activity in any of the tissues tested. BPA induced luc-activity in the liver up to 420 times via both exposure routes. BPA, DEHP and quercetin induced luc-activity in the liver after oral exposure. BPA (50 mg/kg bw IP) also induced luc-activity in the testis, kidneys and tibia. Biomarker-responses in ER-Luc male mice occurred after a single oral dosage to food associated estrogenic compounds at exposure levels 10-10^4 times higher than the established Tolerable Daily Intakes (TDIs) (Chapter 3). However, the estrogenic compounds were given as a single dose, whereas TDI values and also risks associated with dietary exposure can be expected to result from chronic rather than acute exposure. Also in the experimental design of the studies reported in Chapter 3 chemicals were dosed as single compounds whereas in realistic dietary exposure regimens more than one pseudo-estrogen may be present, resulting in combined exposure. Agonist action on the ERα or ERβ may present a similar effect mechanism and therefore one might raise the question whether combined exposure may lead to additive effects. Thus, given the facts that the study described in Chapter 3 did not include chronic exposure and that simultaneous exposure to multiple estrogenic compounds may be a realistic exposure scenario, it was concluded that it remains to be seen whether the margins between the doses shown in the present studies to be without estrogenic effect in the ER-Luc mice and the TDI values reported are indeed sufficiently high to allow the conclusion that these compounds are unlikely to represent a human health risk.
Another factor of importance for the ultimate risk assessment of estrogenic food-associated compounds is related to the fact that exposure to estrogenic compounds during developmental stages could be an important risk factor in developing hormone dependent cancers later in life. Therefore in Chapter 4 seven food-associated estrogenic compounds were studied for their estrogenic effects in fetuses of pregnant female ER-Luc mice. The luc-induction was determined eight hours after a single IP dose or after eight days of daily oral dosing in 0.5 g custard. Three known estrogens, estradiol (E_2), 17α-ethynylestradiol (EE) and EP were used as positive controls at 1 mg/kg bw and DMSO as a solvent control. BPA, NP, *p,p'*-DDE were tested at 50 mg/kg bw, quercetin at 16.6 mg/kg bw, and DIHP, DEHP and DEHA at 100 mg/kg bw. Exposure to E_2, EE and EP resulted in significant luc inductions upon both oral and/or IP dosing in a variety of tissues including liver, tibia and femurs, and upon IP dosing also in the fetuses. BPA, NP, DEHA, DEHP, DIHP, *p,p'*-DDE and quercetin were unable to significantly induce luc-activity in any of the tissues including fetuses. However, unexpectedly, after oral exposure of NP, BPA and DIHP the placental luc-activity was significantly lowered. Thus, in spite of inducing estrogenic activity *in vitro* (Chapter 2), some food-associated compounds were able to reduce the ER-mediated luc-activity in the placenta (Chapter 4), which might be considered an anti-estrogenic effect. This effect might be due to for example a (pseudo)estrogen mediated reduction of aromatase (*5*) or 17β-hydroxysteroidgenase (17βHSD) activity (*6*) known to locally produce E_2 from testosterone (*7*). A reduction in the placental aromatase or 17βHSD inhibition may cause a reduction in the local estrogen levels and thereby explain the reduction in the placental luc activity in ER-Luc mice (Chapter 4). The results in Chapter 4 indicate that at the current levels of exposure to food associated estrogenic compounds, estrogenic effects in the fetus are not expected. However, it was also concluded that the significant luc-reduction in the placenta, should be further studied for its significance for fetal development and relevance for the human situation.

To determine whether the lack of luc-induction in fetuses of exposed pregnant ER-Luc mice reported for several of the food-associated chemicals described in Chapter 4 is due to the inability of these compounds to actually reach the fetal tissues in sufficient amounts, in Chapter 5 of this thesis a study was performed on the amounts of two food associated compounds entering the fetal circulation. The tissue distribution and fate of E_2 and two pseudo-estrogens was studied in pregnant C57black/6J mice after oral exposure during early or late gestation with special emphasis on the amount recovered in the placenta and fetuses. The compounds were dosed as ^{14}C-radioactively labelled compounds ensuring a radioactive dose of 23 µCi/kg bw/day. Doses were 0.1 mg estradiol (E_2)/kg bw/day, used as a reference compound, 10 mg nonylphenol (NP)/kg bw/day or 1 mg *p,p*'-dichlorodiphenyldichloroethylene (*p,p*'-DDE)/kg bw/day. The compounds were fed daily in 0.5 g custard, either during gestation day (GD) 1-8 or GD9-16. Radioactivity was determined in tissues, carcasses, fetuses and excrements. Average total recovery was 41% for E_2, 16% for NP and 54% for *p,p*'-DDE, with the recovery inside the mice amounting to less than 0.2% of the total oral dose for E_2, 0.4% for NP, and 13.8% for *p,p*'-DDE. Most of the compounds was found in faeces and to a lesser degree in urine. The relative distribution of the compounds in the body did not differ between the exposure periods GD1-8 and GD9-16, but markedly differed between the 3 compounds. On average E_2 is recovered mainly in the liver at 23.5 ng/g liver (0.16% of the recovered compound) as was NP 2.2 µg/g liver (0.16%). The presence of 8.7 µg NP/g kidney (0.08%) shows that the kidneys also are a major route of NP-excretion. *p,p*'-DDE was mainly present at levels of 14.3 µg/g in fat (0.82%) and 2.5 µg/g liver (1.9%). The estrogen E_2 did not reach the fetus at a level above the detection limit, NP and *p,p*'-DDE did reach the fetus. Levels of E_2 and NP detected in placenta were relatively high, when compared to those in the fetuses, which might be due to prevention of transport to the fetuses by placental binding proteins. The results of the Chapter 5 reveal the existence of a variable level of the placental barrier function for the (pseudo)estrogens, preventing in some but not all cases high systemic levels of circulating (pseudo)estrogens to actually reach the developing fetus. This indicates that without adequate *in vivo* validation, results from *in vitro* bioassays revealing estrogenic activity of (pseudo)estrogens are not enough to estimate the risk for fetuses.

The effects observed in Chapters 4 and 5 of this thesis are based on effects in rodents, whereas in contrary to humans, rodents do not have a luteo-placental shift (*8*) occurring in humans where the placenta takes over the hormonal production from the corpus luteus after three months. It is interesting to speculate that this could be an important phenomenon resulting in humans but not in rodents in a possible decrease of placental permeability for hormone related compounds, because hormones need no longer to be transported across the placenta. Obviously for adequate rodent-to-human extrapolation of the results presented in the present thesis this aspect needs further elucidation, for example in a species that is known to also have a luteo-placental shift such as guinea pigs.

Concluding remarks and future perspectives

The first effects of endocrine disruptors, such as for example egg shell thinning by DDT (*9*), and abnormal gonad development in alligators in lake Apopka (*10*) were shown in wild-life. In order to test the estrogenicity of certain endocrine disruptors, in recent decades various

in vitro and *in vivo* assays have been developed. Originally the uterotrophic assay, detecting increased uterine weight to assess estrogenic effects, has been used (*11*). Recently, several *in vitro* and *in vivo* reporter gene assays have been developed, allowing rapid quantification and even *in situ* visualization of estrogenic effects by injecting the substrate for the reporter gene (*12,13*). In the present thesis *in vitro* as well as *in vivo* reporter gene assays have been used to assess the estrogenicity of some food-associated compounds. A well-known estrogenic compound included is BPA, known for its estrogenicity, although its low dose estrogenic effects *in vivo* in both experimental animals and man are still a matter of considerable debate (*14,15*). To be able to define the relevance of the results from the *in vitro* and *in vivo* estrogenic activity described in the present thesis for the human situation, data on the human intake as well as information on established tolerable daily intakes (TDIs) of the food associated compounds tested in this thesis are summarized in Table 1. Table 1 also presents an overview of the estrogenic potency of the compounds as measured in this thesis, as well as the margins of safety that can be derived from the estimated human intake and these TDI values.

Table 1: Estrogenicity as determined in the present thesis for 5 well-known food associated compounds, the estimated human intake levels, current TDI levels, and the margins of safety (MOS) between the TDI values and the intake estimates.

Compound	*In vitro* estrogenicity	*In vivo* estrogenicity	Estimated human intake (mg/kg bw/day)	TDI (mg/kg bw/day)	MOS
BPA		+	< 0.001 (*16*); <0.0001 (*17*)	0.01 (*18*)	~10-100
NP	++	-	0.00125 (*19*)	-	-
DEHP	+	-	0.0024 (*20*); 0.003-0.03 (*21*) ; 0.01 (*22*); 0.0032 (*23*) ; 0.03 (*24*)	0.05 (*25,26*) ; 0.037 (*27*)	~ 4.4 – 14.5
DEHA	+	-	0.002 (*22*); 0.00025 (*23*)	0.3 (*28-30*)	150-1200
Quercetin	+	±	0 - 16.6 (Based on 1 g supplement intake per day for a 60 kg person) (*31*)	-	-

From the results presented in Table 1 it can be concluded that the presence of endocrine disrupting compounds in the human diet results in estimated intake levels that are usually 4.4-1200 times below the TDI (Table 1). The results presented in Table 1 also reveal that intake of especially phytoestrogens contributes to human intake of estrogenic compounds also because phytoestrogens like quercetin are presently marketed as food supplements with recommended intakes of 1 gram/day, amounting to 16.6 mg/kg bw/day for a 60 kg person, without adequate safety assessment having been performed (*31*) and TDI/ADI values not being established. Phytoestrogens have been suggested to have beneficial estrogenic effects, via the ERβ (*32,33*) However, these compounds are currently under debate as they may exert proposed positive health effects via ERβ-mediated gene expression (*32,33*), but they are also able to activate ERα-mediated estrogenic effects (*34*). The ultimate outcome of this debate on the ultimate effect on human health of compounds activating both ERα and ERβ remains to be established. The extent to which the outcome of this debate, holds for pseudo-estrogens like BPA, NP and others, present in the human diet at relatively lower levels than the phytoestrogens, also remains to be established. This becomes especially evident when taking into account that for several of these compounds, shown to be estrogenic *in vitro*

(Chapter 2) estrogenicity was not observed in *in vivo* studies with ER-Luc male mice. For example DIHP induced ER-mediated luciferase induction *in vitro* (Chapter 2), whereas *in vivo*, luciferase was not induced in any tissue of male ER-Luc mice (Chapter 3). For other phthalates like DEHP, a small induction in the liver was observed. For these compounds the EU has indicated that replacements should be developed, as phthalates in children's toys should be limited (*29,30*). The results obtained in the present thesis indicate that at the tested concentrations, especially sorbitol-based plasticizers could be safe alternatives for the use of phthalates (Chapter 2).

Based on the findings of the present thesis it was also concluded that in spite of the *in vitro* estrogenicity of various food-born estrogens (Chapter 2), *in vivo* estrogenic effects are not likely expected as estrogenicity *in vivo* is shown at levels 10-10^4 fold higher than the TDI's for humans (Chapter 3). However, as already outlined above, in our studies in Chapter 3 the estrogenic compounds were given as a single dose, whereas TDI values and also risks associated with dietary exposure can be expected to result from chronic rather than acute exposure. Also in the experimental design of the studies reported in Chapter 3 chemicals were dosed as single compounds whereas in realistic dietary exposure regimens more than one pseudoestrogen may be present, resulting in combined exposure. Given that agonist action on the ERα or ERβ may present a similar target and mechanism one might raise the question whether combined exposure may lead to additive effects. Therefore, it remains to be seen whether these margins are sufficiently high. Future experiments are therefore needed that focus on long term and combined exposure.

Another factor that remains to be solved relates to the fact that only in *in vitro* assays the relative ability of compounds to differentially activate ERα or ERβ can be quantified. A specific distribution pattern in the activation of either ERα or ERβ might be detected after exposure of the ER-Luc mice to food packaging associated compounds, when looking specifically at for instance, the lung, prostate and intestines, known to predominantly contain ERβ (*35,36*), and the pituitary, mammary gland, kidney, uterus, testis, liver and ovaries which mainly possess ERα (*35,36*). However, the *in vivo* studies currently available are not able to detect such tissue-specific patterns of induction, mainly because both ER-subtypes induce luc-activity via the same ERE-luc construct and most compounds tested activate both ERs to some extent. Given the fact that both ER subtypes may activate different sets of genes (*37*) *in vivo* validation of this specific aspect of estrogen activity remains an important issue that may be pursued using "omics"-based biomarkers to indentify the genes actually activated in each tissue upon estrogen exposure. This would be an important set of experiments, also because the cellular ERα/ERβ ratio is an important factor determining the influence on induction or inhibition of hormone-related cancers (*38-41*).

The aim of the present thesis was to study the *in vivo* relevance of *in vitro* detected estrogenic effects of food associated compounds with emphasis on prenatal exposure. The results obtained reveal that the extent at which *in vitro* estrogenic effects may result in *in vivo* estrogenicity may vary with the compound under study and should be evaluated on a case by case basis taking differences in absorption, distribution, metabolism and excretion, the intrinsic estrogenic potency of chemicals for either the ERα or ERβ and the differential levels of expression of these receptors in different tissues *in vivo* into account.

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Chapter 7:

Samenvatting en conclusie/discussie/ opmerkingen

Chapter 7:

Samenvatting en concluderende opmerkingen

7

Het doel van dit proefschrift was het bestuderen van de *in vivo* relevantie van *in vitro* aangetoonde oestrogene effecten van voedingsgeassocieerde stoffen, met de nadruk op prenatale blootstelling. De resultaten van het onderzoek laten zien dat *in vitro* oestrogene effecten mogelijk kunnen leiden tot oestrogene effecten *in vivo*, maar dit blijkt per stof en per soort blootstelling te variëren en moet daarom van geval tot geval worden geëvalueerd. Verschillen in absorptie, distributie, metabolisme en excretie zijn enkele redenen waarom *in vitro* oestrogene stoffen *in vivo* andere effecten geven dan *in vitro*. De resultaten in dit proefschrift tonen aan dat beschikbare *in vitro* reporter gen bioassays inzicht kunnen geven in de intrinsieke oestrogene potentie van chemicaliën voor het activeren van de ERα of de ERβ. *In vivo* reporter gen bioassays kunnen laten zien of de oestrogeniteit *in vitro* ook daadwerkelijk resulteert in *in vivo* weefselspecifieke responsen. Ook wordt in dit proefschrift aangetoond dat de *in vitro* bioassays kunnen helpen bij het definiëren van veilige alternatieven voor weekmakers die gebruikt worden in de verpakkingsmiddelenindustrie.

In Hoofdstuk 2 van dit proefschrift is de oestrogene potentie van 21 voedingsgeassocieerde stoffen onderzocht in ERα- of ERβ-getransfecteerde U2-OS (humane osteoblasten zonder endogeen aanwezige oestrogeenreceptoren) reporter gen cellijnen. Zes weekmakers en drie anti-oxidanten waren relatief weinig oestrogeen in de ERα-cellijn. BPA, NP, een weekmaker (tris (2-ethylhexyl) trimellitaat (TEHTM) en twee anti-oxidanten (propylgallaat en gebutyleerd hydroxyanisol) waren oestrogeen in de ERα- en ERβ-cellijn. In verhouding tot E_2, zijn deze stoffen meer oestrogeen in de ERβ-cellijn dan in de ERα-cellijn, wat verklaard kan worden uit het verschil in ligand-bindingsdomeinen van de ERα en de ERβ (*1, 2, 3*). De drie op sorbitol gebaseerde weekmakers activeerden de ERα noch de ERβ en kunnen daarmee mogelijk goede vervangers zijn voor bestaande weekmakers. De voedingsgeassocieerde stoffen dragen bij aan de totale hoeveelheid oestrogene stoffen waar mensen aan worden blootgesteld. Het testen van de specifieke ERα- en ERβ-gemedieerde oestrogene potentie van stoffen is ook van belang in verband met de gesuggereerde positieve effecten van ERβ-gemedieerde responsen en negatieve effecten van ERα-gemedieerde responsen (*4*). Wanneer deze effecten niet worden onderscheiden kan de risicoschatting vertroebelen. Op dit moment kan *in vitro* alleen het verschil in relatieve potentie van chemicaliën voor beide oestrogeenreceptoren worden gekwantificeerd. Echter, deze chemicaliën kunnen verschillen in kinetiek, dat wil zeggen in hun absorptie, distributie, metabolisme en excretiekarakteristieken. De consequenties daarvan kunnen met name in *in vivo*-modellen goed worden bestudeerd. Dat is de reden dat de zeven voedingsgeassocieerde stoffen die in Hoofdstuk 2 *in vitro* oestrogeen bleken te zijn, in Hoofdstuk 3 getest zijn op hun *in vivo* oestrogene effecten in mannelijke muizen met een ER-reporter gen (luciferase) construct.

Mannelijke ER-Luc-muizen werden of IP of oraal gedoseerd en na respectievelijk 8 of 14 uur werd de luciferase (luc) inductie bepaald in verschillende weefsels. 17β-Estradiol 3,17-dipropionaat (EP) in een dosis van 0.3 and 1 mg/kg bw werd gebruikt als positieve controle en DMSO werd gebruikt als oplosmiddelcontrole. De voedingsgeassocieerde oestrogene stoffen werden getest bij niet-toxische doseringen: BPA en NP beide in een dosis van 10 en 50 mg/kg bw), *p,p'*-DDE in een dosis van 5 en 25 mg/kg bw, quercetine in een dosis van 1.66 en 16.6 mg/kg bw, DIHP, DEHP en DEHA alle drie in een dosis van 30 en 100 mg/kg bw. In het algemeen resulteerden IP-doseringen in hogere luc-inducties dan orale doseringen. EP

induceerde de luc-activiteit significant in de lever op een dosisgerelateerde manier met de hoogste inductie van alle geteste stoffen, die 20,000 keer hoger was dan de inductie door de DMSO-controle. NP, *p,p'*-DDE, DEHA en DIHP induceerden de luc-activiteit in geen van de geteste weefsels. BPA induceerde de luc-activiteit in de lever tot maximaal 420 keer via beide toedieningsroutes. BPA, DEHP en quercetine induceerden de luc-activiteit in de lever na orale toediening. BPA (50 mg/kg bw IP) induceerde ook de luc-activiteit in de testis, nieren en tibia. Biomarker-responsen in de ER-Luc mannelijke muizen na een enkele orale dosering, werden meestal geïnduceerd bij blootstellingsniveaus die 10-10^4 keer hoger waren dan de vastgestelde toelaatbare dagelijkse inname (TDI's) (Hoofdstuk 3). Echter de oestrogene stoffen werden in de studie gegeven als een eenmalige dosering, terwijl de TDI-waarden en de risico's die verbonden zijn aan overschrijding van de TDI worden verwacht na chronische blootstelling en niet zozeer na een enkele dosering. Daarnaast geldt dat de stoffen in de opzet in Hoofdstuk 3 als enkelvoudige stof werden gedoseerd, terwijl in werkelijkheid via de blootstelling door voeding meerdere pseudo-oestrogenen tegelijk aanwezig zijn, hetgeen resulteert in een gecombineerde blootstelling. Agonistische activiteit voor een oestrogeen receptor, ERα of ERβ, betekent een vergelijkbaar werkingsmechanisme en daardoor rijst de vraag of gecombineerde bloostelling mogelijk leidt tot additieve effecten. Gegeven het feit dat in de studie, beschreven in Hoofdstuk 3, geen chronische blootstelling is getest en dat gelijktijdige blootstelling aan meerdere oestrogene stoffen waarschijnlijk een meer realistisch blootstellingsscenario is, is geconcludeerd dat het nog niet eenduidig is of de marges tussen de doseringen die in ER-Luc-muizen een oestrogene respons oproepen en de TDI's inderdaad groot genoeg zijn om de conclusie te kunnen trekken dat deze stoffen waarschijnlijk geen risico vormen voor de gezondheid van de mens.

Een andere belangrijke factor bij de risicoanalyse van oestrogene voedingsgeassocieerde stoffen is gerelateerd aan het feit dat blootstelling aan oestrogene stoffen tijdens de prenatale ontwikkeling een belangrijke risicofactor is voor het ontwikkelen van hormoonafhankelijke kankersoorten. In Hoofdstuk 4 zijn daarom de mogelijke oestrogene effecten van zeven voedingsgeassocieerde oestrogene stoffen op foetussen van zwangere ER-Luc vrouwelijke muizen bestudeerd. De luc-inductie is acht uur na een eenmalige IP-dosering of na acht dagen oraal doseren in 0.5 g vanillevla bepaald. Drie bekende oestrogene stoffen, oestradiol (E_2), 17α-ethinyloestradiol (EE) en EP zijn gebruikt als positieve controle, allen bij een dosis van 1 mg/kg bw en DMSO is gebruikt als de oplosmiddelcontrole. BPA, NP, *p,p'*-DDE zijn getest in een dosis van 50 mg/kg bw, quercetine in een dosis van 16.6 mg/kg bw, en DIHP, DEHP en DEHA allen in een dosis van 100 mg/kg bw. Blootstelling aan E_2, EE en EP resulteerde in significante luc-inducties na zowel orale als IP-doseringen in een verscheidenheid aan weefsels, waaronder lever, tibia en femurs, en na IP-dosering ook in de foetussen. BPA, NP, DEHA, DEHP, DIHP, *p,p'*-DDE en quercetine waren niet in staat om de luc-activiteit in weefsels, inclusief de foetussen, significant te induceren. De significante verlaging van de luc-activiteit in de placenta na orale blootstelling aan NP, BPA en DIHP was onverwacht. Ondanks het induceren van oestrogene activiteit *in vitro* (Hoofdstuk 2), waren sommige voedselgeassocieerde stoffen in staat de ER-gemedieerde luc-activiteit in de placenta te verlagen (Hoofdstuk 4), wat mogelijk gezien kan worden als een anti-oestrogeen effect. Dit effect kan bijvoorbeeld veroorzaakt worden door een (pseudo)oestrogeen-gemedieerde reductie van de aromatase- (*5*) of 17β-hydroxysteroidgenase (17βHSD)-

activiteit (*6*), waarvan bekend is dat ze locaal E_2 produceren uit testosteron (*7*). Een reductie of remming van placentair aromatase of 17βHSD kan daardoor een verlaging van locale oestrogeenniveaus veroorzaken en daarmee en reductie van de placentaire luc-activiteit in ER-Luc-muizen verklaren (Hoofdstuk 4). De resultaten in Hoofdstuk 4 geven aan dat bij de huidige blootstellingsniveaus aan voedingsgeassocieerde oestrogene stoffen, oestrogene effecten in de foetus niet worden verwacht. Er is echter, ook geconcludeerd dat de significante luc-reductie in de placenta verder bestudeert moet worden waarbij speciaal gekeken moet worden naar de mogelijke gevolgen van dit effect voor de foetale ontwikkeling en de relevantie van het effect voor de humane situatie.

Om vast te stellen of het afwezig zijn van luc-inductie door voedingsgeassocieerde oestrogene stoffen in de foetussen van blootgestelde drachtige ER-Luc-muizen, zoals beschreven in Hoofdstuk 4, komt doordat deze stoffen de foetussen niet kunnen bereiken, is in Hoofdstuk 5 een onderzoek uitgevoerd naar het transport van twee van deze stoffen naar de foetus. De weefselverdeling met speciale aandacht voor de foetus en het lot van E_2 en nonylphenol (NP) en *p,p'*-dichloordiphenyldichloorethyleen (*p,p'*-DDE) is onderzocht in drachtige C57black/6J-muizen na orale toediening tijdens de vroege of late dracht. De gedoseerde stoffen werden toegediend als radioactief ^{14}C-gelabelde stoffen, met een radioactiviteit van 23 µCi/kg bw/dag. De doseringen waren 0.1 mg estradiol (E_2)/kg bw/dag, gebruikt als referentiestof, 10 mg NP/kg bw/dag of 1 mg *p,p'*-DDE/kg bw/dag. De stoffen werden dagelijks gegeven in 0.5 g vanillevla, van dag 1-8 van de dracht (GD1-8), of van dag 9-16 van de dracht (GD9-16). De radioactiviteit is bepaald in weefsels, karkassen, foetussen en uitwerpselen. De gemiddelde totale recovery was 41% voor E_2, 16% voor NP en 54% voor *p,p'*-DDE, waarvan in de muizen minder was dan respectievelijk 0.2%, 0.4% en 13.8% werd teruggevonden. Het merendeel van de stoffen werd gemeten in de fecaliën en iets minder in de urine. De relatieve distributie van de stoffen verschilde niet tussen de beide blootstellingsperiodes GD1-8 en GD9-16, maar was erg verschillend tussen de drie stoffen. Over het algemeen werd E_2 met name teruggevonden in de lever in een hoeveelheid van 23.5 ng/g lever (0.16% van de teruggevonden hoeveelheid E_2) en deze waarde bedroeg 2.2 µg/g lever (0.16%) voor NP. De aanwezigheid van 8.7 µg NP/g nier (0.08%) toont aan dat de nieren een belangrijke rol spelen bij de excretie van NP. *p,p'*-DDE werd met name in het vet teruggevonden; 14.3 µg/g (0.82%) en 2.5 µg/g (1.9%) in de lever. De oestrogene stof E_2 bereikte de foetus niet boven de vastgestelde detectielimiet, NP en *p,p'*-DDE bereikten de foetus wel. De niveaus van E_2 en NP die werden gevonden in de placenta waren relatief hoog, vergeleken met de niveaus van deze stoffen in de foetus, wat mogelijk duidt op een bescherming van de foetus door vermindering van het transport van de stoffen naar de foetus als gevolg van hun binding aan placentaire bindingseiwitten. De resultaten van Hoofdstuk 5 laten het bestaan van een variabele placentaire barrièrefunctie voor (pseudo)oestrogenen zien die in sommige, maar niet alle gevallen, kan voorkomen dat hoge concentraties van circulerende (pseudo)oestrogenen de foetus bereiken. Dit impliceert dat zonder adequate *in vivo*-validatie, resultaten van *in vitro*-bioassays die oestrogeniteit van (pseudo)oestrogenen aantonen, niet genoeg informatie leveren voor het inschatten van het risico voor de foetus.

De effecten gezien in Hoofdstuk 4 en 5 van dit proefschrift zijn gemeten in knaagdieren, terwijl in tegenstelling tot de humane situatie, knaagdieren geen luteo-placentaire shift hebben (*8*). De luteo-placentaire shift in mensen is het moment waarop de placenta de

hormonale productie van het corpus luteum overneemt na drie maanden zwangerschap. Het is interessant om te speculeren dat dit fenomeen in mensen, maar niet in knaagdieren, mogelijk leidt tot een vermindering in placentaire permeabiliteit, omdat hormonen niet langer over de placenta hoeven te worden getransporteerd. Het is duidelijk dat voor een adequate knaagdier-humane extrapolatie van de resultaten in dit proefschrift een verdere opheldering van dit aspect noodzakelijk is. Dit zou kunnen in een diersoort waarvan ook bekend is dat deze een luteo-placentaire shift heeft, zoals de cavia.

Concluderende opmerkingen en toekomst perspectieven

De eerste effecten van hormoonverstorende stoffen, zoals de afname in eischaaldikte van eieren door DDT (*9*), en abnormale genitaliënontwikkeling bij alligators in lake Apopka (*10*) werden aangetoond in de natuur. Om de oestrogeniteit van hormoonverstorende stoffen te testen zijn de afgelopen jaren vele *in vitro*- en *in vivo*-assays ontwikkeld. Van oudsher is de uterotrofe-assay veel gebruikt, die een toename in uterusgewicht als eindpunt voor oestrogene werking heeft (*11*). Recentelijk zijn meerdere *in vitro*- en *in vivo*–reportergen-assays ontwikkeld, die snelle kwantificering en zelfs *in situ*-visualisering van oestrogene effecten mogelijk maken door het injecteren van het substraat voor het reporter gen (*12,13*). In dit proefschrift zijn zowel *in vitro*- alsook *in vivo*-reportergen-assays gebruikt om de oestrogeniteit van voedingsgeassocieerde stoffen te bepalen. Een bekende oestrogene stof die is meegenomen is BPA, hoewel de oestrogeniteit van deze stof bij lage doseringen *in vivo* in dierexperimenteel- en humaan onderzoek in twijfel wordt getrokken (*14,15*). Om de relevantie van de *in vitro*- en *in vivo*-oestrogene activiteit zoals beschreven in dit proefschrift voor de humane situatie te bepalen, zijn bekende data van humane inname en vastgestelde toelaatbare dagelijkse inname (TDIs) van de voedingsgeassocieerde stoffen, zoals getest in dit proefschrift, samengevat in Tabel 1. Tabel 1 geeft ook een overzicht van de oestrogene potentie van de stoffen gebruikt in dit proefschrift, evenals veiligheidsmarges die kunnen worden afgeleid van de humane inname en de TDI-waarden.

Tabel 1: De oestrogeniteit zoals bepaald in dit proefschrift voor 5 bekende voedings-geassocieerde stoffen, de geschatte humane inname niveaus, de huidige TDI waarden, en de veiligheidsmarges (MOS) tussen de TDI waarden en de geschatte inname niveaus.

Stof	*In vitro* oestrogeniteit	*In vivo* oestrogeniteit	Geschatte humane inname (mg/kg bw/dag)	TDI (mg/kg bw/dag)	MOS
BPA		+	< 0.001 (*16*); <0.0001 (*17*)	0.01 (*18*)	~10-100
NP	++	-	0.00125 (*19*)	-	-
DEHP	+	-	0.0024 (*20*); 0.003-0.03 (*21*) ; 0.01 (*22*); 0.0032 (*23*) ; 0.03 (*24*)	0.05 (*25,26*) ; 0.037 (*27*)	~ 4.4 – 14.5
DEHA	+	-	0.002 (*22*); 0.00025 (*23*)	0.3 (*28-30*)	150-1200
Quercetine	+	±	0 - 16.6 (Gebaseerd een 1 grams supplement-inname per dag voor een persoon van 60 kg) (*31*)	-	-

Uit de resultaten van Tabel 1 kan worden geconcludeerd dat de aanwezigheid van hormoonverstorende stoffen in het humane dieet resulteert in geschatte innameniveaus die meestal 4.4-1200 maal onder de TDI liggen (Tabel 1). De resultaten in Tabel 1 tonen ook aan dat vooral de inname van phyto-oestrogenen bijdraagt aan de humane inname van oestrogene stoffen, ook omdat phyto-oestrogenen zoals quercetine in de markt worden gezet als voedingssupplement met aanbevolen dagelijkse inname van 1 gram/dag. Dit leidt tot een inname van 16.6 mg/kg bw/dag voor een persoon van 60 kg, zonder dat er een adequate risicobeoordeling heeft plaatsgevonden (*31*) en zonder dat er een TDI/ADI-waarde is vastgesteld. Van phyto-oestrogenen wordt gesuggereerd dat ze met name positieve oestrogene effecten via de ERβ mediëren (*32,33*). Ook dit staat nog niet vast, omdat phyto-oestrogenen niet alleen zogenoemde positieve gezondheidseffecten via ERβ-gemedieerde genexpressie induceren (*32,33*), maar omdat ze ook in staat zijn ERα-gemedieerde oestrogene effecten te induceren (*34*). De uitkomst van het debat over deze gecombineerde activatie moet nog worden vastgesteld. Het debat over stoffen die zowel ERα als ERβ activeren en hun effecten op de humane gezondheid, betreft ook pseudo-oestrogenen zoals BPA, NP en anderen. De effecten van deze stoffen, die aanwezig zijn in het humane dieet in lagere concentraties dan phyto-oestrogenen, moeten ook nog worden vastgesteld. Dit wordt in het bijzonder duidelijk omdat voor bepaalde stoffen die oestrogeen bleken *in vitro* (Hoofdstuk 2), oestrogeniteit niet werd aangetoond in *in vivo*-studies met ER-Luc mannelijke muizen. DIHP induceerde bijvoorbeeld wel ER-gemedieerde luciferaseinductie *in vitro* (Hoofdstuk 2), terwijl *in vivo*, luciferase in geen enkel weefsel van mannelijke ER-Luc werd geïnduceerd (Hoofdstuk 3). Andere ftalaten zoals DEHP, induceerden luciferase in de lever. Voor deze stoffen heeft de EU gesteld dat er vervangers moeten worden ontwikkeld, zeker omdat ftalaten in kinderspeelgoed zoveel mogelijk moeten worden gelimiteerd (*29,30*). De resultaten in dit proefschrift tonen aan dat bij de geteste concentraties, in het bijzonder de op sorbitol gebaseerde weekmakers veilige alternatieven zijn voor ftalaten (Hoofdstuk 2).

De resultaten uit dit proefschrift suggereren dat -ondanks de *in vitro*-oestrogeniteit van bepaalde voedingsgeassocieerde oestrogenen (Hoofdstuk 2)- *in vivo*-oestrogene effecten niet waarschijnlijk zijn omdat de oestrogeniteit zoals *in vivo* is aangetoond, vaak is gevonden bij concentraties die 10-10^4 keer hoger zijn dan de TDI's voor deze stoffen (Hoofdstuk 3). Echter zoals hierboven al beschreven, zijn in Hoofdstuk 3 de oestrogene stoffen eenmalig gedoseerd, terwijl TDI-waarden en risico's die geassocieerd worden met blootstelling via het dieet, vaak gekoppeld zijn aan (het ontbreken van) effecten na chronische blootstelling. In het experimentele ontwerp van Hoofdstuk 3 werden de stoffen bovendien als enkelvoudige verbinding gegeven, terwijl een realistisch dieet resulteert in een gecombineerde blootstelling aan meer dan één pseudo-oestrogeen. Gezien het feit dat een agonistische werking op de ERα of ERβ kan resulteren in een vergelijkbaar effect in het lichaam, kan men de vraag stellen of gecombineerde blootstelling kan leiden tot additieve effecten. Het valt om die reden nog te bezien of de huidige veiligheidsmarges groot genoeg zijn. Toekomstige experimenten zijn nodig om het effect van langdurige en gecombineerde blootstellingen aan oestrogene stoffen te onderzoeken.

Een andere factor die nog niet opgelost is, is het feit dat in *in vitro* assays alleen de relatieve activiteit voor de ERα of ERβ gekwantificeerd kan worden. Een specifiek weefseldistributiepatroon in het activeren van ERα of ERβ kan mogelijk gedetecteerd

worden na blootstelling van voedingsgeassocieerde stoffen in ER-Luc-muizen, omdat van bijvoorbeeld de long, prostaat en darmen bekend is dat zij met name ERβ bevatten (*35,36*), terwijl de hypofyse, borstweefsel, nieren, uterus, testis, lever en eileiders bijvoorbeeld vooral ERα bevatten (*35,36*). De huidige *in vivo*-studies zijn niet in staat deze weefselspecifieke patronen te detecteren, waarschijnlijk doordat beide ER-subtypes de luc-activiteit induceren via hetzelfde ERE-luc-construct en dat de meeste stoffen beide ERs in ieder geval gedeeltelijk activeren. Omdat beide ER-subtypes mogelijk verschillende genen activeren (*37*) is *in vivo*-validatie van dit specifieke aspect van oestrogene activiteit een belangrijke zaak, die mogelijk kan worden aangepakt via een "omics"-gebaseerde biomarkerbenadering, waarbij de genen geïdentificeerd worden die daadwerkelijk in een bepaald weefsel geactiveerd worden na blootstelling van het dier aan een oestrogene stof. Dit is een belangrijke set van experimenten, want de ERα/ERβ-ratio in weefsels is een belangrijke factor die van invloed is op de mogelijke inductie of inhibitie van hormoongerelateerde kankersoorten (*38-41*).

Het doel van dit proefschrift was om de *in vivo*-relevantie van *in vitro*-gedetecteerde oestrogene effecten van voedingsgeassocieerde stoffen te bestuderen, met nadruk op prenatale blootstelling. De resultaten laten zien dat *in vitro*-oestrogene effecten mogelijk resulteren in *in vivo*–oestrogeniteit of anti-oestrogeniteit, maar dat dit varieert per stof en per studie en van geval tot geval zal moeten worden geëvalueerd, rekening houdend met de absorptie, distributie, metabolisme en excretie, de intrinsieke oestrogene potentie van stoffen voor de ERα of ERβ en de verschillende niveaus van expressie van deze beide receptoren in verschillende weefsels.

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Chapter 8:

Miscellaneous

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List of abbreviations

ADI	Acceptable Daily Intake
BHA	Butylated Hydroxyl Anisole
BPA	Bisphenol A
DCC	Dextran Coated Charcoal
DDE	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethylene
DDT	Dichlorodiphenyltrichloroethylene
DEHA	Di (2-ethylhexyl) adipate
DEHP	Di (2-ethylhexyl) phthalate
DIHP	Diisoheptyl phthalate
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethylsulfoxide
E_2	17β-estradiol
EC_{50}	Concentration at which 50% of the effect is reached
EE	17α-ethynylestradiol
EEF	Estradiol Equivalence Factor
EEQ	Estradiol Equivalents
EP	17β-estradiol-3,17-dipropionate
ERα/β	Estrogen receptor alpha/beta
ERE	Estrogen Responsive Elements
ER-Luc	Estrogen receptor reporter gene (luciferase)
ESO	Epoxidized Soy Bean oil
FCS	Fetal Calf Serum
NP	Nonylphenol
IF	Induction Factor
IP	Intraperitoneal
IsDH	Isosorbide di-*n*-hexanoate
IsDiB	Isosorbide diiso butyrate
IsDEH	Isosorbide di 2-ethylhexanoate
Luc	Luciferase gene
PBS	Phosphate Buffered Saline
pM	picoMolar
REC	Relative estrogenic concentration
RLU	Relative Light Units
TDI	Tolerable Daily Intake
TEHTM	Tris (2-ethylhexyl) trimellitate
U2-OS	human osteoblastic cell line

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Marcel

Curriculum vitae

Marcel Gerben Rudolph ter Veld werd op 23 juli 1979 geboren in Zuidvelde (Norg, Drenthe). In 1997 behaalde hij zijn atheneum diploma op het Dr. Nassau college te Assen. In datzelfde jaar begon hij aan de opleiding Voeding en Gezondheid aan de Wageningen Universiteit. Een afstudeervak werd uitgevoerd onder begeleiding van Dr. G. M. Alink en Prof. Dr. Ir. I. M. C. M. Rietjens op de afdeling Toxicologie. Een tweede afstudeervak werd uitgevoerd op het RIKILT onder begeleiding van Dr. L. A. P. Hoogenboom en Dr. Ir. T. F. H. Bovee. In 2002 behaalde hij zijn diploma. Aansluitend startte hij zijn promotieonderzoek op de afdeling Toxicologie onder begeleiding van Prof. Dr. Ir. I. M. C. M. Rietjens en Prof. Dr. A. J. Murk, waarvan de resultaten in dit proefschrift beschreven zijn. Tijdens zijn promotie heeft hij zitting gehad als expert op het gebied van dierproeven en alternatieven in de Dierexperimenten commissie van de Wageningen Universiteit, sinds 2006 tevens als vice-voorziter. Sinds september 2006 is hij werkzaam als groepsleider van het dierexperimententeam en study director algemene toxicologie studies in de pre-klinische fase bij Schering-Plough (voormalig Organon).

List of publications

Hester van der Woude, Marcel G. R. ter Veld, Natasja Jacobs, Paul T. van der Saag, Albertinka J. Murk, Ivonne M. C. M. Rietjens. The stimulation of cell proliferation by quercetin is mediated by the estrogen receptor. *Molecular nutrition and food research, 49(8): 763, 2005.*

Veld, M.G.R. ter; B. Schouten; J. Louisse, D.S. van Es; P.T. van der Saag ,I.M.C.M Rietjens and Murk, A.J. Estrogenic potency of food-packaging-associated plasticizers and antioxidants as detected in ERα and ERβ reporter gene cell lines. JAFC, 54(12): 4407, 2006.

Veld, M.G.R. ter, E. Zawadzka, J.H.J. van den Berg, P. van der Saag, Rietjens, I.M.C.M. and Murk, A.J. Food associated estrogenic compounds induce estrogen receptor mediated luciferase gene expression in transgenic male mice. *Chem Bio Int 174: 126, 2008.*

Veld, M.G.R. ter, E. Zawadzka, Rietjens, I.M.C.M. and Murk, A.J. Estrogenicity of food associated estrogenic compounds in the offspring of female estrogen receptor-reporter gene mice upon oral and IP maternal exposure. (*Submitted)*

Veld, M.G.R. ter; J. Louisse; Rietjens, I.M.C.M. and Murk, A.J. Tissue distribution and fate of the estrogenic compounds estradiol, nonylphenol and *p,p'*-dichlorodiphenyldichloroethylene in pregnant C57black mice. (*In preparation*)

Training and Supervision Plan

Overview of conferences attended during the PhD period.

Poster presentation, SOT, NC, USA	2007
Oral presentation PhD days, NVT, Wageningen, The Netherlands	2006
Poster presentation SETAC, The Hague, The Netherlands	2006
Oral presentation PhD days, NVT, Oss, The Netherlands	2005
Oral presentation SETAC, Lille, France	2005
Poster presentation PhD days, NVT, Veldhoven, The Netherlands	2004

Overview of courses attended during the PhD period.

Organ Toxicology; Utrecht (PET)	2005
Mutagenesis & Carcinogenesis; Leiden (PET)	2005
Legal & Regulatory Toxicology; Leiden (PET)	2005
Discussion techniques (Gespreksvaardigheden); WUR/OWU	2005
Time planning and projectmanagement; WUR, PE&RC Graduate School	2004
Environmental Toxicology; WUR (TOX-20304)	2004
Reproductive Toxicology; Utrecht (PET)	2004
Medical and Forensic Toxicology; Utrecht (PET)	2003
Toxicological Risk Assessment; Wageningen (PET)	2003
Organizing and supervizing thesis work; WUR/OWU	2003
Writing and presenting scientific papers; WUR, Mansholt Graduate School	2002
Safe handling radioactive materials (Radiation course 5B); WUR/Larenstein	2002

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