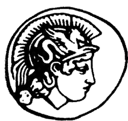
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Ability of xeno- and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotropic activity

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Abstract

The function of the uterus is regulated by female sex steroids and it is, therefore, used as the classical target organ to detect estrogenic action. Uterine response to estrogens involves the activation of a large pattern of estrogen-sensitive genes. This fact offers the opportunity to analyze the estrogenic activity of xeno- and phytoestrogens, and the mechanisms of their molecular action by a correlation of the uterotropic activity and their ability to modulate the expression of estrogen-sensitive genes. We have analyzed the expression of androgen receptor (AR), progesterone receptor (PR), estrogen receptor (ER), clusterin (CLU), complement C3 (C3), and GAPDH mRNA in the rat uterus following oral administration of ethinylestradiol (EE), bisphenol A (BPA), *o,p′*-DDT (DDT), *p*-tert-octylphenol (OCT) and daidzein (DAI). A significant stimulation of the uterine wet weight could be observed after administration of all the substances. The activity of all analyzed compounds to stimulate uterine weight was low in comparison to EE. DDT has the highest activity to stimulate uterine weight whereas BPA and DAI turned out to be less potent. The analysis of gene expression revealed a very specific profile of molecular action in response to the different compounds which cannot be detected by judging the uterotropic response alone. A dose dependent analysis revealed that C3 mRNA is already modulated at doses where no uterotropic response was detectable. Although DAI and BPA were very weak stimulators of uterine growth, these substances were able to alter the expression of AR, ER and C3 very strongly. Based on these investigations the analyzed compounds can be subdivided into distinct classes: First, compounds which exhibit a similar gene expression fingerprint as EE (e.g. OCT); second, compounds exhibiting a significant uterotropic activity, but inducing a pattern of gene expression different from EE (e.g. DDT); and third, compounds like BPA and especially DAI which exhibit a very low uterotropic activity, but nevertheless modulate the expression of estrogen-sensitive genes. These findings strongly suggest that the fingerprint of uterine gene expression is a very sensitive tool to investigate estrogenicity of natural and synthetic compounds and offers the possibility to get information in regard to the molecular mechanisms involved in the action of the respective compounds. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Xenoestrogens; Phytoestrogens; Uterus; Bisphenol A; Complement C3; Clusterin; Daidzein

1. Introduction

Over the last decades numerous chemicals have been identified which bind to the estrogen receptor and/or trigger mechanisms of estrogen action [1,2]. Although the activity of most of these environmental estrogens is low compared to endogenous or synthetic estrogens, like 17β-estradiol or ethinylestradiol, dietary or environmental exposure scenarios have been described which led to the detection of significant quantities of these substances in human urine [3] and tissue samples

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[4]. Epidemiological data suggest that the consumption of some of these environmental estrogens may be beneficial, for example, by offering protection against breast and prostate cancer [5,6], whereas the others may act as endocrine disrupters which as a consequence could affect the endocrine system and may cause developmental [7,8] and reproductive disturbances [9,10]. For these reasons there is the urgent need to characterise the hormonal activity of compounds with the ability to bind to the estrogen receptor and to elucidate the molecular mechanisms of their action. Recently, a considerable amount of evidence has been accumulated which demonstrates that the molecular mechanisms involved in the action of estrogens and particularly estrogen-like compounds, are more complex as believed initially. The discovery of mechanisms like cross talk [11], protein interactions between estrogen receptors and transcription factors [12] and new receptors like the estrogen receptor β [13–15] have to be considered if the estrogenic activity of the so-called environmental estrogens is investigated.

Due to this complexity of the molecular mechanisms involved in the uterine-responsiveness to estrogen-like compounds, a powerful in vivo test has to be used to evaluate the entire complexity of a possible response. A classical in vivo tool for the prediction of estrogenicity is the uterotropic assay [16]. In this test system the stimulatory activity for uterine weight in ovariectomized or juvenile female mice or rats, is determined. In this report we demonstrate a considerable analytical upgrade of this assay which enables us to increase considerably the sensitivity and the information obtained from uterotropic assay in respect of molecular mechanisms. The uterine response to estrogens involves the activation of a large pattern of estrogen sensitive genes. Analysis of the pattern of expressed genes offers the opportunity to quantify the estrogenic activity of a substance and in parallel to elucidate the molecular mechanisms of its action. The panel of analyzed genes in the uterus include androgen receptor (AR) [17,18], progesterone receptor (PR) [19,20], estrogen receptor (ER) [21,22], clusterin (CLU) [23], complement C3 (C3) [24], and GAPDH [25] following oral administration of ethinylestradiol (EE), the xenoestrogens bisphenol A (BPA), *o,p′*-DDT (DDT), *p*-tert-octylphenol (OCT) and the phytoestrogen daidzein (DAI) (Fig. 1) in ovariectomized female DA/Han rats. Whereas the transcription of C3 and PR is regulated by estrogen-responsive elements in the promotor [27,28], CLU expression is modulated by steroid hormones via an AP-1 response element [29]. The molecular mechanisms involved in the regulation of AR, ER and GAPDH gene expression by estrogens, are so far unknown. Finally, the selected genes display a distinct sensitivity to estradiol. Therefore, the analysis of these genes in combination with the uterine growth response provides information not only in regard to the activity, but also in regard to the molecular mechanisms of the action of the administered compounds.

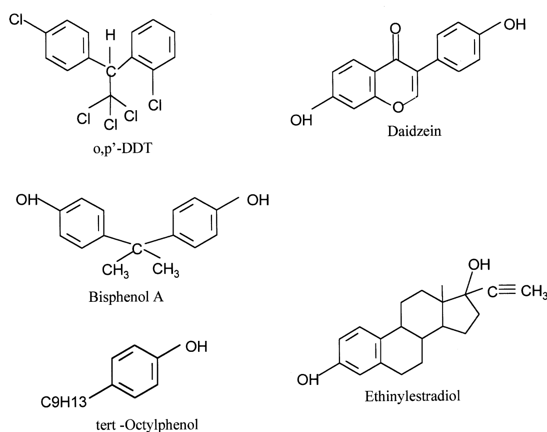

Fig. 1. Structures of compounds investigated in this study.

2. Material and methods

2.1. Animals

DA/Han rats were selected for these experiments since the RUCA-I cell line established from an endometrial adenocarcinoma of DA/Han rats [30] significantly responds to estrogen [31] as well as xenoestrogen treatment by an alteration of gene expression [32,33]. Juvenile female DA/Han rats (130 g) were obtained from Moellegard (Moellegard Breeding and Research, Lille Skensved, Denmark) and were maintained under controlled conditions of temperature (20°C ± 1), relative humidity (50–80%) and illumination (12 h light, 12 h dark). All rats had free access to standard rat diet (SSniff R10-Diet, SSniff GmbH, Soest, Germany) and water.

2.2. Compounds

Ethinylestradiol (17α-Ethynyl-1, 3, 5[10]-estriene-3, 17β-diol;19-Nor-1,3, 5[10],17a-pregnatrien-20yne-3,17-diol) was provided by Schering AG (Berlin, Germany), *p*-tert-octylphenol was provided by Hüls AG (Marl, Germany), bisphenol A (4, 4-Isopropylidenediphenol) was provided by Bayer AG (Leverkusen, Germany), daidzein (4′,7-Dihydroxyisoflavone) was obtained from Biomol (Hamburg, Germany), *o,p′*-DDT (2-(ortho-chlorophenyl)-2-(para-chlorophenyl)-1,1,1-trichloroethane) was obtained from Promchem GMBH (Wesel, Germany).

2.3. Treatment

Animals were ovariectomized (ovx). After 14 days of endogenous hormonal decline the animals were treated 3 days pre-operative by gastric tube with the respective compounds. The animals were randomly allocated to treatment and vehicle groups ($n = 6$). EE (100 μg/kg BW), OCT (5, 50 and 200 mg/kg BW), BPA (5, 50 and 200 mg/kg BW), DDT (20, 100 and 500 mg/kg BW) and DAI (10, 100 and 500 mg/kg BW) were dissolved in dimethylsulfoxide (DMSO). Compounds were given in a total volume of 1 ml/animal. Animals were sacrificed by decapitation after light anaesthesia with CO_2 inhalation. Uterus wet weight was determined and the uteri were snap frozen in liquid nitrogen for RNA preparation.

2.4. RNA isolation and complementary DNA synthesis

Total cytoplasmic RNA was extracted from the cells according to the guanidinium–thiocyanate–CsCl method described by Sambrook et al. [34]. DNA-free RNA was obtained by treatment with ribonuclease-free deoxyribonuclease I in the presence of placental ribonuclease inhibitor for 30 min at 37°C. After phenol–chlorofom (extraction and ethanol precipitation, RT's were performed using the Superscript preamplification system (Life Technologies, Gaithersburg, MD).

2.5. Northern blotting

Northern Blot analysis was performed as described by Sambrook et al. [34]. Hybridisation was carried out according to the protocol for rapid hybridisation (Stratagene, Heidelberg, Germany). For reference hybridisation a probe coding for 18 S RNA was used. Autoradiography was performed by exposing Kodak X-omat AR film (Eastman Kodak, Rochester, NY, USA) to the nylon filters at −80°C in presence of an enhancing screen. Autoradiograms were analyzed by densitometry.

2.6. Oligonucleotide primers for PCR reactions

Based on the cDNA sequences available at the EMBL databank, the following specific primer pairs were designed: GAPDH sense primer 5′-TGAAGGTCGGTGTGAACGGATTT-3′; GAPDH antisense primer 5′-CACAGTCTTCTGAGTGG-CAGTGAT-3′; PR sense primer 5′-CATGTCAGTG-GACAGATGCT-3′; PR antisense primer 5′-ACTTCAGACATCATTTCCGG-3′; AR sense primer 5′-GACCAGATGGCAGTCATTCAG-3′; AR antisense primer 5′-CAGCTCTCTTGCAATAGGCTG-3′; ER sense primer 5′-TGACCAACCTGGCAGA-CAGG-3′; ER antisense primer 5′-GCCTTTGTTACTCATGTGCC-3′; C3 sense primer 5′-CAGCCCGAAGAGTGCCAGTAGTC-3′; C3 antisense primer 5′-CCATCCTCCTTTCCATCAACTGC-3′; CLU sense primer 5′-CCCTTCTACTTCTGGAT-GAA-3′; CLU antisense primer 5′-GAACAGTCCA-CAGACAAGAT-3′; Cytochrome c oxidase subunit I sense primer; 5′-CGTCACAGCCCATGCATTCG-3′; antisense primer 5′-CTGTTCATCCTGTTCCAGCTC-3′. Cytochrome c oxidase subunit I (1A) was used as reference gene. Primers were synthesised by MWG Biotech AG (Ebersbach, Germany). PCR-products were sequenced to verify their identity and homology to corresponding cDNA sequences in the EMBL databank.

2.7. Semiquantitative PCR

Semiquantitative PCR was performed according to the method described by Murphy et al. [35] and modified by Knauthe et al. [22]. To normalise signals from different RNA samples, cytochrome c oxidase subunit I (1A) was coamplified as internal standard. Amplification reactions were stopped before leaving the exponential phase. Amplification was performed using a Perking-Elmer/Cetus 9600 thermal cycler (Norwalk, CT). Thermus flavus polymerase (0.5 U; Biozym, Hess. Oldendorf, Germany), dNTPs (dATP, dGTP, dCTP and dTTP, 200 μmol/ each); and the respective oligonucleotid primers (500 ng each) were added to an amount of first strand cDNA equivalent to 200 ng total RNA. The reaction volume was adjusted to 50 μl using 1 × PCR buffer [50 mM Tris–HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$ and 2.5 mM $MgCl_2$]. Amplification cycles comprised a 1-min step at 94°C for denaturation, a 1-min step at 58°C for annealing and a 1-min step at 72°C for elongation. Reaction products were separated on 1 × Tris borad EDTA-6% polyacrylamide gels and detected by ethidium bromid staining.

2.8. Statistical analysis

For the statistical analysis of the uterine weight we used two-way analysis of variance followed by pairwise comparison of selected means using the pooled within-group variance comparisons. The statistical significance of the PCR–mRNA measurements was determined using the Nemenyi Test. The criterion for significance was set at $p \leq 0.05$.

3. Results

3.1. Uterotropic response to the oral administration of BPA, DDT, OCT and DAI

The uterotropic response to the administration of

selected xeno- and phytoestrogens was assessed by a three-day uterotropic assay. Ovariectomized female DA/Han ($n = 6$) rats were treated for 3 days pre-operative (p.o.) with the xeno-estrogens BPA (5, 50, 200 mg/kg/day), OCT (5, 50, 200 mg/kg/day), DDT (20, 100, 500 mg/kg/day), the phytoestrogen DAI (20, 100, 500 mg/kg/day) and EE (100 μg/kg/day). The uterine wet weight was evaluated. Table 1 shows the uterine wet weights following administration of increasing amounts of the used compounds tested. The uterus wet weight was stimulated in a dose-dependent manner following treatment with DDT (100 and 500 mg/kg/day). After administration of 200 mg/kg/day OCT a significant stimulation was detectable. A significant but very low stimulation could be detected following treatment with 200 mg/kg/day BPA and 500 mg/kg/day DAI.

3.2. Validation of the semiquantitative PCR assay

In former investigations [22,26] we have demonstrated that our semiquantitative PCR-system is a fast, highly reproducible and sensitive procedure to analyse gene expression. To validate our semiquantitative PCR-system in regard to the uterine gene expression we have analyzed the mRNA expression of CLU, a highly expressed estrogen-sensitive gene in the uterus by northern blotting and semiquantitative PCR. The expression of CLU mRNA was analyzed in the uteri of those test groups which responded with a significant increase in uterine wet weight to the administration of the respective substances. As shown in Fig. 2, almost identical results could be obtained using northern blotting and PCR.

Table 1
Uterus wet weights after three day administration of *o,p*-DDT, tert-octylphenol, bisphenol A and daidzein in relation to ethinylestradiol

Treatment	Dose (mg/kg/BW day)	Uterus wet weight (mg/kg/BW)
OVX	–	451 ± 52
EE	0.1	1751 ± 208[a]
	5	440 ± 88
OCT	50	493 ± 74
	200	857 ± 73[a]
	5	437 ± 56
BPA	50	440 ± 34
	200	562 ± 33[a]
	10	430 ± 43
DAI	100	492 ± 50
	500	548 ± 49[a]
	10	493 ± 96
DDT	100	897 ± 77[a]
	500	1116 ± 185[a]

[a] Significantly different from corresponding vehicle control at $p \leq 0.05$.

3.3. Regulation of the analyzed genes

In order to correlate the increase of the uterine wet weight following administration of the tested substances with their ability to modulate the expression of estrogen-sensitive genes, the mRNA expression of AR, ER, PR, GAPDH, CLU and C3 was analyzed in the uteri of those test groups which responded with a significant increase in uterine wet weight. The analysis of gene expression revealed a far more complex profile of action of the analyzed compounds than the data from the uterotropic assay would have predicted. The results are shown in Fig. 3. Treatment with all compounds led to a down-regulation of ER and AR mRNA. The expression of PR mRNA in the uterus was slightly induced by EE, whereas a significant decrease of PR mRNA expression was observed following administration of DDT and BPA. PR mRNA expression was not affected after administration of OCT and DAI.

Clusterin mRNA was strongly down-regulated by EE and DDT. Administration of OCT and DAI resulted in a smaller down-regulation of CLU mRNA expression whereas administration of BPA had no effect. The expression of C3 mRNA was enhanced by all compounds tested. Interestingly, the administration of compounds like BPA and DAI also caused a strong increase of C3 mRNA expression whereas the wet weight of the uterus was only slightly increased. GAPDH mRNA expression was induced by EE and OCT. Administration of DAI and DDT led to a significant decrease in GAPDH expression, whereas administration of BPA had no effect.

The observed complexity of effects of the tested phyto- and xenoestrogens on the expression of the analyzed genes are summarised in a so-called gene expression fingerprint. This fingerprint offers the unique opportunity to compare regulatory effects caused by phyto- and xenoestrogens directly to those caused by EE. As shown in Fig. 4, the gene expression fingerprint of OCT is most similar to that of EE whereas BPA displays the most different fingerprint in comparison to that of EE.

3.4. Correlation of dose-dependency of gene expression and uterotropic response

The gene expression fingerprint has demonstrated that among the analyzed genes, C3 is the most sensitive estrogen-responsive parameter in the uterus. To correlate the sensitivity of the uterine gene expression to the uterotropic response, we have analyzed the dose dependent mRNA expression of C3 following administration of the respective compounds. Fig. 5 shows the dose-dependent mRNA expression of C3 after oral administration of EE, OCT, BPA, DDT and DAI. A clear dose-dependent increase of C3 expression after

administration of DDT was visible which is in keeping with the uterotropic response. A good correlation of C3 mRNA expression and uterotropic response was also observed after administration of OCT and BPA. Interestingly, the response of C3 mRNA expression after the dose-dependent administration of DAI differs from the uterotropic response. The C3 mRNA expression was strongly stimulated at DAI doses where no stimulation of uterine weight is visible.

4. Discussion

In this study we have correlated the uterotropic activity of phyto- and xenoestrogens following oral administration to their ability to modulate the expression of estrogen-sensitive uterine genes. It has to be pointed out that analysis of protein levels of the analyzed genes would be a more physiologic endpoint than the analysis of mRNA expression. However, only the analysis of mRNA expression using PCR techniques allows us to compare the expression of a large amount of different genes in an acceptable period of time. Our investigations reveal substance specific effects which may on the one hand reflect differences in their estrogenic activity, but may indicate on the other hand the differences in their molecular mechanisms of action. As shown in Table 1, the uterotropic activity of all analyzed phyto- and xenoestrogens was very low after oral administration when compared to the activity of the reference compound EE. However, some significant stimulation of the uterine wet weight was detected after administration of all compounds (Table 1) at the highest doses. In regard to the uterotropic response, the most potent xenoestrogen appeared to be DDT (Fig. 2). This effect is surprising, when compared to in vitro data obtained in the RUCA-I cells, since neither did DDT possess a high binding affinity to the ERα of RUCA-I cells [33], nor did it significantly stimulate estrogen-dependent gene expression in this cells (Vollmer, unpublished data). The molecular gene expression fingerprint of DDT reveals many similarities with that of EE, nevertheless there are also some striking differences. In contrast to that of EE, DDT fingerprint is not able to stimulate the expression of PR mRNA and GAPDH mRNA (Fig. 4); on the contrary, the expression of these genes is even diminished. A reduced PR mRNA expression could also be observed following administration of BPA. A down regulation of PR mRNA has been described as response to its own ligand, to pure antiestrogens [36], progesterone [37] and to androgens [38]. In addition it is known that, for instance, the DDT-metabolite p′p-DDE exert antiandrogenic activity [39]. Sohoni et al. [40] have demonstrated that not only DDT but also BPA is able to act like antiandrogens in yeast transactivation test systems. Therefore, these effects may be caused by an interaction of these metabolites with the androgen receptor, but there is also the possibility that progesterone receptor is involved in particular since these substances could not compete for binding of ^{3}H-mibolerone to the AR of LNCap cells (Vollmer, unpublished results).

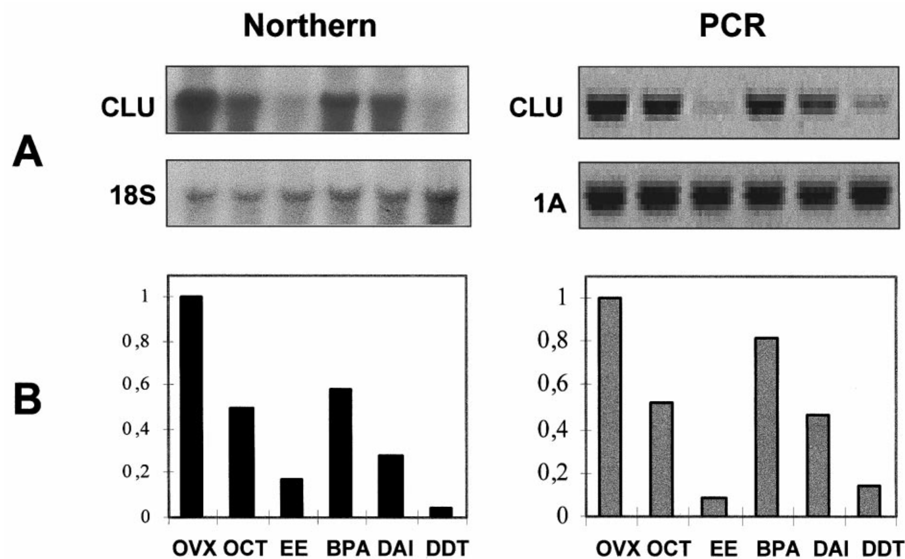

Fig. 2. Analysis of uterine clusterin (CLU) mRNA expression after administration of daidzein (DAI 500 mg/kg/day), *o,p′*-DDT (DDT 500 mg/kg/day), *p*-tert-octylphenol (OCT 200 mg/kg/day), bishenol A (BPA 200 mg/kg/day) or ethinylestradiol (EE 100 µg/kg/day). Correlation of northern blot and semiquantitative PCR. OVX = ovariectomized vehicle treated animal. A Representative gel pattern. B Densitometric analysis. The intensity of the bands was scanned. The quotient reference gene/CLU was calculated. The quotient of the expression of the reference gene/CLU mRNA in the ovariectomized vehicle treated animal group was defined as 1.

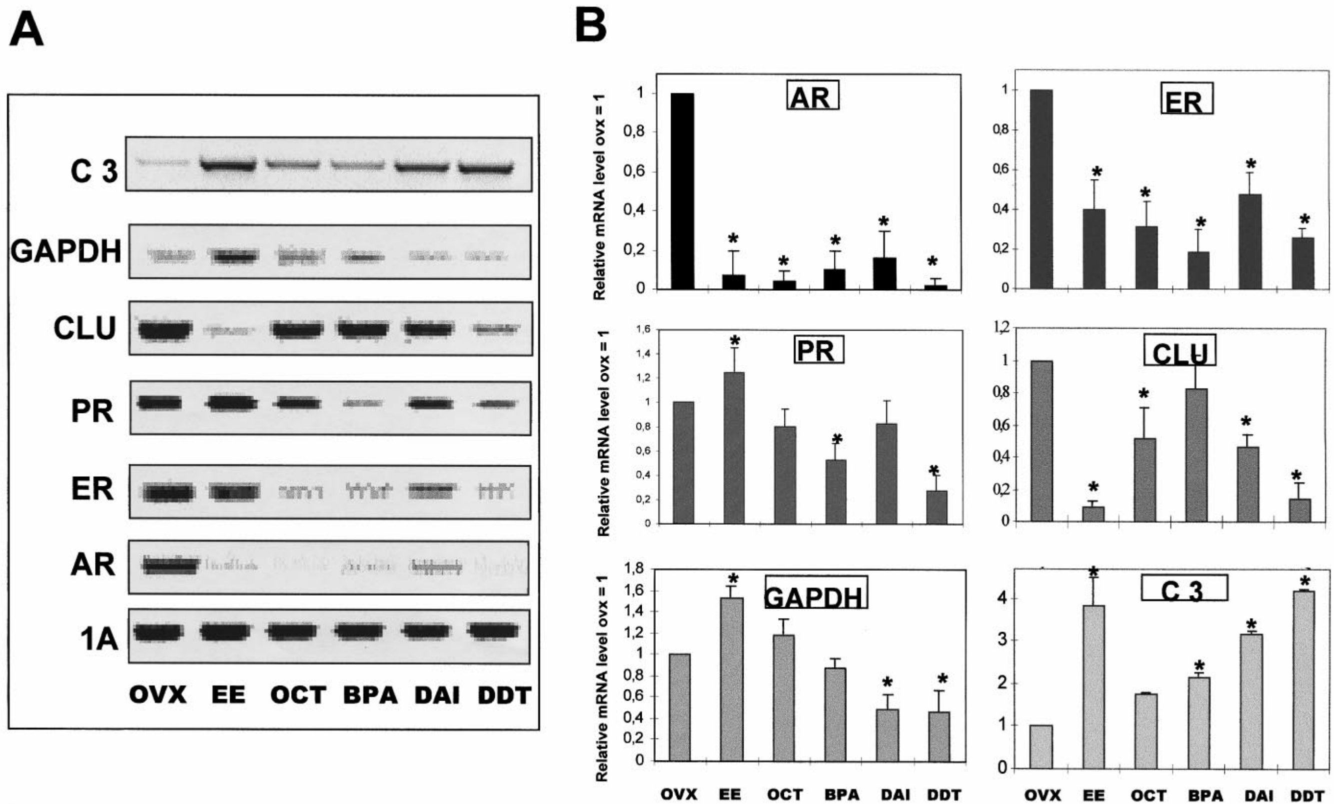

Fig. 3. Analysis of uterine clusterine (CLU), androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), GAPDH and C3 mRNA expression after treatment with daidzein (DAI 500 mg/kg/day), *o,p′*-DDT (DDT 500 mg/kg/day), tert-octylphenol (OCT 200 mg/kg/day), bisphenol A (BPA 200 mg/kg/day) and ethinylestradiol (EE 100 μg/kg/day). Analysis by semiquantitative PCR. OVX = ovariectomized vehicle treated animal. For the analysis of each treatment group the pooled RNA of $n = 6$ uteri was analyzed. cDNA synthesis and semiquantive PCR analysis was performed independently four times. The results are shown as mean ± SD. The mRNA expression of ovariectomized vehicle treated animal group was defined as 1. Statistical significant differences ($p \leq 0.05$) of the mRNA expression in regard to the ovariectomized vehicle treated animal group are indicated by ∗.

	EE	OCT	DDT	DAI	BPA
AR	⇓⇓⇓	⇓⇓⇓	⇓⇓⇓	⇓⇓⇓	⇓⇓⇓
ER	⇓⇓	⇓⇓	⇓⇓	⇓⇓	⇓⇓
PR	⇑	—	⇓⇓	—	⇓
CLU	⇓⇓⇓	⇓⇓	⇓⇓⇓	⇓⇓	—
GAPDH	⇑⇑	⇑	⇓	⇓	—
C 3	⇑⇑⇑	⇑⇑	⇑⇑⇑	⇑⇑⇑	⇑⇑

Fig. 4. Gene expression fingerprint. The regulatory tendencies of the uterine clusterine (CLU), androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), GAPDH and C3 mRNA expression after administration of daidzein (DAI 500 mg/kg/day), *o,p*-DDT (DDT 500 mg/kg/day), tert-octylphenol (OCT 200 mg/kg/day), bisphenol A (BPA 200 mg/kg/day) and ethinylestradiol (EE 100μg/kg/ day) are indicated. ⇑ = faint increase, ⇑⇑ = medium increase, ⇑⇑⇑ = strong increase of mRNA expression, ⇓ = faint decrease, ⇓⇓ = medium decrease, ⇓⇓⇓ = strong decrease of mRNA expression, — = no change. Differences to EE are indicated by the grey colour.

Therefore, it might be important to further investigate to find out whether these compounds and their metabolites possess significant binding affinity to the progesterone receptor.

The uterotropic activity of OCT is low in comparison to the activity of DDT. This is in agreement with the published data, which describe alkylphenoles as weak stimulators of the uterine growth in prepubertal rats [41]. In contrast to its uterotropic activity the gene expression fingerprint of OCT always matched with that of EE, indicating a substantial estrogen-like activity of this substance at the molecular level when compared to that of EE. A single difference in the gene expression pattern of OCT is due to its failure to increase the PR mRNA expression in the uterus. It has also to be stressed that in EE-treated animals the increase of PR mRNA in the total uterus tissue is rather faint. Therefore, we believe that the failure of OCT to induce PR RNA expression documents a lower estrogenic activity of the compound rather than a different mode of action at the molecular level.

In our investigations the xenoestrogen BPA showed a very low uterotropic activity following oral administration. Recently, it has been demonstrated that BPA is active in very high doses (400–800 mg/kg) in uterotropic assays with immature AP rat, both using either oral or subcutaneous administration [42] of the compound. The low but significant increase of uterine wet weight observed in our studies following oral administration of a daily oral dose of 200 mg/kg BPA is in agreement with these findings. However, despite the low uterotropic activity of BPA, there are clear indications of an estrogen-like action of this compounds if the uterine gene expression is examined. BPA is able to down-regulate uterine AR and ER mRNA expression and to induce C3 expression.

The phytoestrogen DAI appears to be a very interesting substance. DAI has been described as a rather potent [32] or a weak estrogen [15] depending on the in vitro test system and the analyzed experimental parameter. In keeping with our data the uterotropic activity of DAI has previously been described to be low [43]. However, at the molecular level DAI acts like a potent estrogen which is in agreement with the in vitro studies [32]. DAI is capable of down-regulating CLU, AR and ER mRNA expression significantly and, more interestingly, it induces the expression of the C3 gene

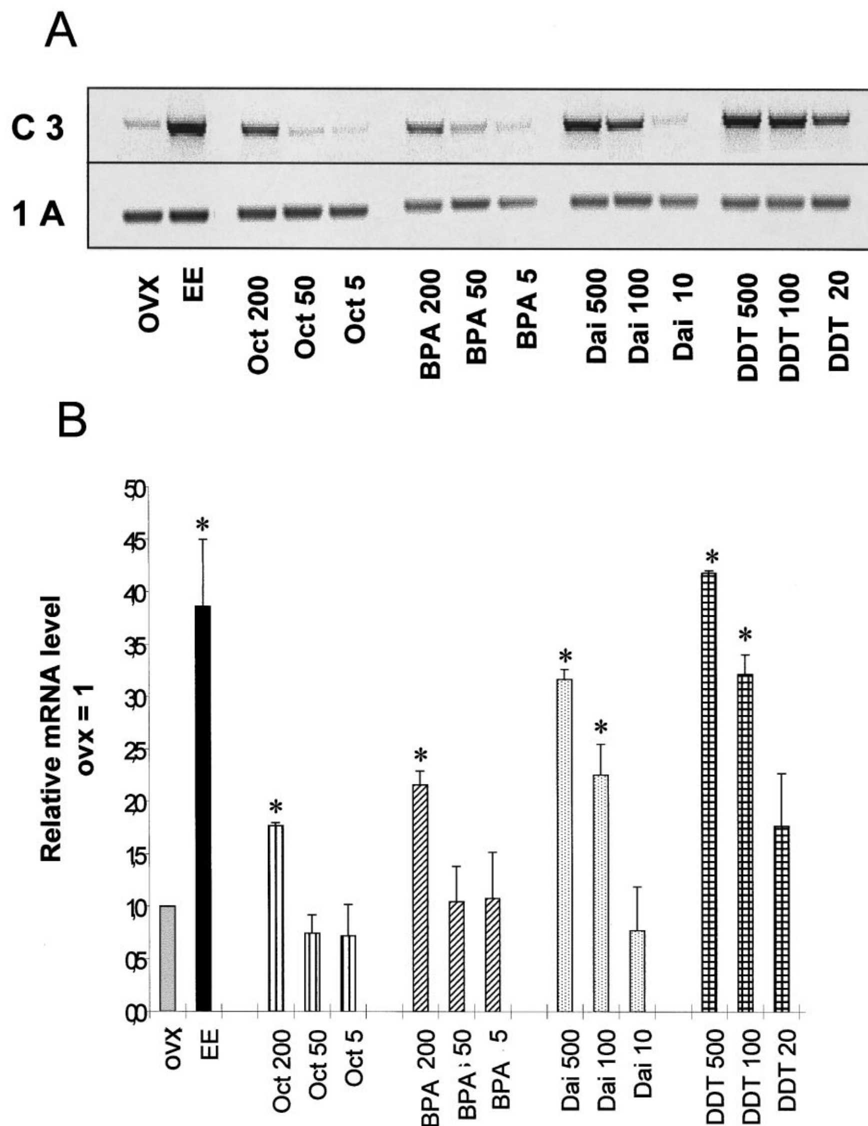

Fig. 5. Dose-dependent stimulation of C3 gene expression after administration of EE, OCT, BPA, DAI and DDT. A. Representative mRNA patterns. B. Amounts of C3 mRNA. The amount of PCR products was determined by densitometric analysis. For the analysis of each treatment group the pooled RNA of $n = 6$ uteri was analyzed. cDNA synthesis and semiquantive PCR analysis was performed independently four times. The results are shown as mean $\pm$ SD. The mRNA expression of ovariectomized vehicle treated animal group was defined as 1. Statistical significant differences ($p \leq 0.05$) of the mRNA expression in regard to the ovariectomized vehicle treated animal group are indicated by*.

in a dose-dependent manner. This finding is important because it demonstrates that DAI exerts a clear estrogen-like action on the uterus at the molecular level without stimulation of the uterine weight. It would be very interesting to further investigate the action of this compound in other estrogen-sensitive tissues like the mamma and the uterus. In our opinion the discrepancy between the uterotropic activity of BPA and DAI and their ability to modulate the uterine gene expression reflect the complex molecular mechanisms which are involved in the regulation of gene expression by these compounds. Recently Das et. al (1998) have demonstrated that interaction of a compound with the nuclear ER is not an absolute requirement for the induction of specific estrogen-like effects in reproductive target tissues [19]. There is also the possibility that estrogen-like effects could be induced by substances via an indirect action on the pituitary gland [44] or the non-genomic activation of signal transduction cascades, like increase of intracellular calcium levels [45,46], or activation of MAP-kinases [47,48] or not even require ERα or ERβ, as recently shown for methoxychlor [49].

In summary, we could subdivide the analyzed substances in distinct categories according to their properties (Fig. 4). The first group comprises substances which possess some uterotropic activity and, on the molecular level mimic the action of EE. These group of compounds can be classified as very weak estrogens. An example for this is OCT. The second group of substances is characterised by a relatively strong estrogenic activity but with a different gene expression fingerprint compared to that of EE. Such compounds are capable of inducing estrogen-regulated genes in the uterus, but they also show differences in their action on the expression of individual genes. An example for such a compound is DDT. The third class of compounds shows a low estrogenic activity but nevertheless is able to modulate the uterine gene expression. These compounds, for example BPA or DAI, may act estrogen like but the molecular mechanisms involved in their action may differ from those of natural estrogens [50]. It is possible that the action of these compounds involves the crosstalk with other signal transduction pathways [51], the binding to other steroid hormone receptors [15] or direct action on the pituitary gland like recently demonstrated for BPA [44].

In regard to risk assessment our data demonstrate that the combination of the classical uterotropic assay with an analysis of the expression of estrogen-sensitive genes in the rat uterus represents a very sensitive and powerful tool to identify substances with estrogenic activity. We could demonstrate that the alteration of the expression of specific uterine genes, especially C3, is a more estrogen-sensitive parameter than the increase of uterine wet weight. In regard to this it has to be mentioned that the C3 gene responds after a single treatment with estradiol (data not shown). Analysis of C3 mRNA expression after a single treatment therefore could be a time saving procedure to analyse the estrogenicity of substances. In addition, this approach of using a complex analysis of uterine gene expression provides clues to the understanding of the molecular mechanisms involved in the action. In our view this combined knowledge, represents an improvement in regard to the complex process of risk assessment of distinct substances.

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