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Peptides as sensors of oestrogen receptor conformations**Novel modes of oestrogen receptor agonism and antagonism by hydroxylated and chlorinated biphenyls, revealed by conformation-specific peptide recognition patterns**

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Key words

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Abstract

Because of the concern about environmental chemicals with oestrogenic and anti-oestrogenic effects, there is a need to construct biosensors for classifying such chemicals according to their effect on oestrogen receptor conformation. The conformation of the ligand binding domains (LBD) of oestrogen receptor- α and - β determine their transcription regulation activity. Some ligands, *i.e.*, the natural oestrogen oestradiol, induce an active conformation allowing interaction with co-activators. In contrast, antagonists like ICI 162,780, because of their bulky side chains, do not allow an α -helix twelve positioning compatible with co-activator binding. Another type of oestrogen receptor-ligand interactions, termed "passive antagonism", was first defined by X-ray crystal structure analysis of receptors in complex with the side chain-less 5,11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC). We have now used the ability of peptides selected from phage-displayed peptide libraries to bind conformation-specifically to oestrogen receptor- α and - β LBDs to analyse conformations induced by THC and a group of chlorinated biphenyls and their aryl-hydroxylated metabolites, suspected of being environmental chemical disruptors. In oestrogen receptor- β , THC defined a "passive antagonist" peptide recognition pattern, which was also induced by several antagonistic hydroxylated biphenyls, while a clearly different peptide recognition pattern was induced by their chlorinated agonistic counterparts. In oestrogen receptor- α , THC induced a conformation similar to that induced by oestriol and other oestrogen receptor- α agonists, which, as evaluated by site-directed mutagenesis, have a functionally important interaction with oestrogen receptor- α residue His524. We conclude that the peptide recognition pattern can be used to used classify suspected environmental endocrine disruptors according the oestrogen receptor- α and - β conformations they induce.

1. Introduction

The physiological effects of endogenous and synthetic oestrogens are mediated by the oestrogen receptors (ERs), ER α and ER β . Ligand binding to the ERs initiates a series of molecular events culminating in the activation or repression of transcription of target genes (McKenna et al., 2002). Transcriptional regulation requires direct interaction of ligand-occupied ERs with its cognate DNA target site (oestrogen responsive elements, EREs) in the regulatory regions of oestrogen-responsive genes (Egea et al., 2000). In addition, ligand-induced conformational changes of the ligand binding domain (LBD) confer further specificity upon the hormonal response by allowing recognition of co-activators and are key events permitting a variety of natural and synthetic ligands to elicit different responses.

The most prominent ligand-induced conformational change is a repositioning of α -helix 12 (Fig. 1). In general, different positionings of α -helix 12 and associated changes in other helices result in different co-regulator binding surfaces and hence underlie the different physiological effects of agonists and antagonists (Brzozowski et al., 1997; Pike et al 1999; Pike et al 2001; Shiau et al., 2002) (Fig. 1). When oestradiol (E2), the natural oestrogen, binds to the ERs' LBD, the phenolic hydroxyl group of its A-ring nestles between the LBD's α -helices 3 and 6 and makes direct hydrogen bonds to the carboxylate of Glu353 and the guanidinium group of Arg394 (using the ER α template residue numbering system). The 17 β hydroxyl of E2's D-ring makes a hydrogen bond with His524 in α -helix 11. As a consequence of these interactions, α -helix 12 lies over the ligand-binding cavity and forms, together with α -helices 3 and 5, a groove-binding site for the Leu-Xaa-Xaa-Leu-Leu motif of receptor co-activators like p160/SRC-1 (Kong et al., 2005; Cheskis et al 2003). In contrast, the side chain of partial agonists like 4-hydroxytamoxifen (OHT) partially pushes α -helix 12 away, forcing it into a conformation in which it binds to the co-activator binding site and reduces the affinity for the co-activator. The side chain of pure antagonists like ICI 182,780 binds directly to the co-activator binding site, preventing co-activator binding and causing α -helix 12 to be completely disordered (Brzozowski et al., 1997; Pike et al 1999; Pike et al 2001). Surprisingly, some compounds with similar antagonistic structure, such as raloxifene, are so-called selective oestrogen receptor modulators or SERMs, *i.e.*, they are agonists in some tissues but antagonists in other. For example, raloxifene displays anti-oestrogenic action in both uterus and breast, but retains agonistic activity in bone (reviewed in Regitz-Zagrosek et al., 2007).

Recently, an additional type of ER-ligand interactions has been discovered, which is referred to as "passive antagonism" or "antagonism without a side chain" (Shiau et al., 2002). Measuring transactivation

activity by transfection experiments, the compound 5,11-cis-diethyl-5,6,11,12-tetrahydrochrysen-2,8-diol (THC) has been found to be an agonist for ER α and an antagonist for ER β (Meyers et al., 1999; Sun et al., 1999). However, unlike other antagonists, it does not possess a bulky side chain. X-ray crystal structure analysis has shown that THC induces an ER α conformation similar to that induced by E2, but induces a non-productive ER β conformation of key residues in the ligand binding pocket, which stabilises an inactive conformation of α -helix 12 and disfavours the co-activator-binding LBD conformation (Shiau et al., 2002) (Fig. 1).

Recently, there has been an increasing concern about environmental chemicals that may interfere with oestrogenic action and adversely affect reproduction of humans and wildlife. These compounds, known as environmental endocrine disruptors, encompass a wide range of substances including natural products, pesticides, pharmaceuticals and industrial chemicals (Matthews et al., 2000; Scippo et al., 2004). There is therefore a need for biosensors which are able to evaluate informatively and fast ER α and ER β conformations induced by such compounds. We have recently begun utilising the ability of peptides isolated from phage-displayed peptide libraries to recognise specific ER conformations (Paige et al., 1999). Each conformation is recognised by a unique combination of peptides, defining a specific peptide recognition pattern. We characterised ER α and ER β conformations induced by suspected environmental endocrine disruptors among pesticides (Sumbayev et al., 2005) and tested various nanotechnological assays based on peptide-receptor binding (Mukhopadhyay et al., 2005; Hansen et al., 2007). Based on these data, it seems that peptide recognition patterns may serve as sensors of ER conformations.

In the present study, we use the peptide recognition patterns to study a group of chlorinated biphenyls. In mammals, these compounds undergo dechlorinative or demethylative hydroxylation. Some of these chemicals have been shown to have either ER agonistic or antagonistic properties in cell culture experiments (Shiau et al., 2002; Andersen et al., 2002; Dees et al 1997; Matthews et al., 2000a, b). Since none of the compounds have large side chains, we have studied the conformation they induce in the ERs with special reference to the concept of "passive antagonism" or "antagonism without a side chain".

2. Materials and methods

2.1. Materials

The following materials were purchased from the indicated suppliers: streptavidin (Calbiochem, San Diego, CA, USA); full length ER α and ER β (Panvera, Madison, WI, USA); DNA cassettes, primers, and biotinylated oligonucleotides corresponding to vitellogenin ERE (biotin-GATCTAGGTCACAGTGACCTGCG – forward and biotin-GATCCGCAGGTCAGTGTGACCTA – reverse) (DNA Technology, Aarhus, Denmark); E2, E3, and OHT (Sigma, St. Louis, MO, USA); various pesticides (Dr. Ehrenstorfer, Augsburg, Germany); mouse anti-M13 monoclonal antibody conjugated with horseradish peroxidase (HRP) and anti-GST goat antibody (Amersham Biosciences, Hillerød, Denmark); glutathione (GSH) agarose (Molecular Probes, Eugene, OR, USA); biotinylated GSH (Biopeptide, San-Diego, CA, USA); Maxisorp™ microtitre plates (Nunc, Roskilde, Denmark); *E. coli* BL21 (Stratagene, La Jolla, CA, USA); materials for SPR studies (BIAcore, Uppsala, Sweden). The methoxychlor/DDT hydroxylated derivative HPTE was synthesised as described (Gaido et al., 1999). All other materials were of the best grade commercially available.

2.2. Ligand binding assay

Determination of the K_D values for binding of ligands to ER α -LBD was performed by a modification of a previously described procedure (Matthews et al., 2000), using the ability of various concentrations of the ligands in question to displace a low concentration of tritiated E2 from GST-ER α -LBD. Biotinylated GSH (100 pmol per well) was incubated in streptavidin-coated wells for 1 h, followed by a 1 h incubation with GST-ER α -LBD (3 pmol per well) and a 2 h incubation with 0.8 nM tritiated E2 and various concentrations of the ligands in question in non-radioactive form in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20). All incubations were at 37°C. After the incubations, the supernatants were taken for liquid scintillation counting, the wells washed 5 times with TBST-buffer, and bound tritiated E2 eluted by 50 mM Tris, pH 8.0, 150 mM NaCl, 100 mM GSH and subjected to liquid scintillation counting. The K_D values for binding of the ligands in question were determined from plots of [bound E2]/[free E2] versus the logarithm to the concentration of the ligand, using the SigmaPlot software for fitting the data points to the equation:

$$[\text{bound E2}]/[\text{free E2}] = [R]_T / ([\text{free E2}] + K_{D, E2} + (K_{D, E2}/K_{D, \text{ligand}}) \cdot [\text{ligand}]) + C$$

in which $[R]_T$ is the total receptor concentration, $K_{D, E2}$ is the K_D value for binding of E2, $K_{D, ligand}$ is the K_D value for the ligand in question, $[ligand]$ is the concentration of the ligand, and C is a constant.

2.3. Fusion phage and D1D2 fusion protein

M13 phage displaying ER-binding peptides were prepared by ligating oligonucleotide cassettes coding for each peptide into Sfil-digested fUSE5 vector followed by production of fusion phage in *E. coli* DH-5 α cells (Scott, 1990). The created fusion phage was validated by DNA sequencing of PCR products generated with the primers M13for (5'-TTTCGACACAATTTATCAGG-3') and M13back (5'-TGAATTTTCTGTATGAGGTTTTG-3') and Taq DNA polymerase (Invitrogen, Denmark). The PCR-products were purified (Qiaquick PCR Purification Kit, Qiagen, Germany) and sequenced with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Hillerød, Denmark) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA).

D1D2 fusion protein displaying each of the ER-LBD binding peptides were prepared by ligating oligonucleotide cassettes coding for each peptide into Sfil-digested D1D2 fused to pET vector followed by plasmid production in *E. coli* DH-5 α cells. The created fusion proteins were validated by DNA sequencing of PCR products generated with the respective primers and Taq DNA polymerase (Invitrogen, Denmark). The PCR-products were purified (Qiaquick PCR Purification Kit, Qiagen, Germany) and sequenced with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Hillerød, Denmark) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA). Fusion proteins were purified from BL21 (DE3)pLysS *Escherichia coli*. Expression was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to log-phase cultures followed by incubation for 3 h at 37°C. Cells were lysed by sonication on ice in a buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 μ M ZnCl₂, 0.5% NP-40, 0.5 mM dithiothreitol, 1 mM phenyl-methyl-sulfonyl-fluoride and 0.3 μ g/ml lysozyme. The D1D2 fusion proteins were purified on a Ni-nitrilo-triacetic acid (Ni-NTA) column, followed by gel filtration through Sephadex G75 column.

2.4. Site-directed mutagenesis and purification of the GST-ER α -LBD fusion protein and 6 \times His-tagged ER α -LBD

The H524A mutation was introduced into the GST-ER α -LBD expression vector, kindly provided by Dr. M. Brown (Boston, MA, USA) (Halachmi et al., 1990), using the QuickChange Site-Directed Mutagenesis Kit

(Stratagene, La Jolla, CA, USA) with the primers 5'-GGCATGGAGGCGCTGTACAGCATGAAGTGCAAGAACGTGGTGCCC-3' and 5'-GGGACCACGTTCTTGCACTTCATGCTGTACAGCGCCTCCATGCC-3' (Sumbayev et al., 2005). The introduction of the mutation was validated by DNA sequencing as described above. GST-ER α -LBD and GST-ER α -LBD-H524A fusion proteins were purified from *E. coli* BL21 cells transformed with the corresponding expression vectors. Expression was induced by adding IPTG to log-phase cultures followed by incubation for 2 h at 37°C. Cells were lysed by sonication on ice in a buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 μ M ZnCl₂, 0.5% NP-40, 0.5 mM dithiothreitol, 1 mM phenyl-methyl-sulfonyl-fluoride and 0.3 μ g/ml lysozyme. The GST-ER α -LBD (molecular weight is 64.5 KDa) fusion proteins were purified by chromatography on a GSH-agarose column (Matthews et al., 2000). The LBD migrated as a single, M_r xxxxx, > 95% pure band in Coomassie Blue stained SDS-PAGE (data not shown)

2.5. Phage ELISA with full length ERs and with GST-ER α -LBD

The ELISAs were performed at 37°C in TBST buffer. Two ELISA formats were used. In ELISA-format I, wells of a microtitre plate coated with streptavidin were incubated with biotinylated vitellogenin ERE (2 pmol per well) for 1 h, followed by monomeric, full length ER α or ER β (2 pmol per well) for 1 h. In ELISA-format II, the streptavidin-coated wells were incubated with biotinylated GSH (100 pmol per well) for 1 h followed by GST-ER α -LBD (3 pmol per well) for 1 h. In both ELISA formats, fusion phage ($\sim 10^{10}$ colony forming units per well) was allowed to bind to the immobilised ER or GST-ER α -LBD, respectively, in the presence of the appropriate ER ligand as indicated for each experiment. The bound fusion phage was detected with a HRP-conjugated anti-M13 monoclonal antibody and a peroxidase reaction (*ortho*-phenylenediamine/H₂O₂, Kem-En-Tek Diagnostics, Copenhagen, Denmark). Colour development was measured by a microplate reader as absorbance at 492 nm (Sumbayev et al., 2005).

2.6. SPR Analyses

The measurements were performed using a BIACORE X instrument. All experiments were performed at 25°C in a binding buffer of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.005% Tween 20, 1 mM sodium ascorbate (Mukhopadhyay et al., 2005). Peptides fused to the D1D2 protein were immobilized on CM5 chips to a density of 3526 ± 612 RU per mm² (3 different experiments per peptide), using the standard

BIAcore amine-coupling kit. Samples with various concentrations of GST-ER α -LBD or full length ER β were injected over the peptide surface for 120 s, using a flow rate of 40 μ l/min. After discontinuation of injection, dissociation of bound ER was followed for another 10 min. A control surface with no peptide immobilised was used as a reference. One μ M ER ligand was present in the running buffer during the whole experiment (before, during and after injection). The chip surfaces were regenerated down to background level by applying two or three 1-min pulses of 10 mM glycine, pH 2.0.

The dissociation rate constants (k_{-1} values) were estimated from semilogarithmic plots of the relative amount of ER complex bound to the chip versus time during the dissociation phase, using the equation

$$[\text{bound ER}] = A \cdot \exp((-k_{-1}) \cdot t) + B$$

in which A and B are constants corresponding to maximum and background binding levels, respectively. The association rate constants (k_1 values) were determined by following the association phase with 500 nM ER. The apparent association rate constant ($k_{\text{ass,app}}$) was determined by a fit to the equation

$$[\text{bound ER}] = A \cdot (1 - \exp((-k_{\text{app}}) \cdot t)) + B$$

The true k_1 values were then determined from the equation

$$k_1 = (k_{\text{ass,app}} - k_{-1}) / [\text{ER}]$$

the k_{-1} value being determined from the dissociation phase in the very same run. The K_D values for peptide ER binding were calculated as the ratios between the dissociation rate constants (k_{-1} values) and the association rate constants (k_1 values).

2.7. Statistical analysis

Each experiment was performed three independent times. Unless otherwise indicated, statistical analysis was done using the two-tailed Student's *t*-test with a significance level of $p < 0.01$.

3. Results

3.1. Ligand-receptor binding assay

Our goal was to study the conformational states of the ER-LBD induced by the synthetic oestrogens, polychlorinated and hydroxylated biphenyls as well as isoflanone-derived phytoestrogens. The trans-activation activity or trans-activation inhibitory ability of all compounds except DCBP and DHBP were characterised previously (Andersen, et al., 2002, Gaido, et al., 1999). In order to estimate the ligand concentrations needed to saturate the receptors, we determined the K_D values for their binding to ER α wt and ER α H524A. A typical experiment is shown in Fig. 2, and a summary of all results in Table 1.

The estimated K_D values vary widely, from 1 nM for E2 to 350 nM for methoxychlor. The H524A mutation decreased the affinity of most ligands to the receptor. Interestingly, the mutation had no effect on receptor affinity of 2,4-dichlorodiphenyl-dichloroethylene (DDT) and 2,2'-dichlorobenzophenone (DCBP), both of which are polychlorinated and lack hydroxyl groups and therefore unable to make hydrogen bonds to His524. The mutation had a slight effect on the affinity of methoxychlor, although this compound is also unable to make hydrogen bonds.

For the studies below, we routinely used a ligand concentration of 1 μ M, which will lead to full or almost full saturation in all cases. We also ensured, by scrutinising the data, that the variability of the peptide recognition patterns could in no case be explained by a lack of saturation of receptor with ligand.

3.2. Ligand-dependent peptide recognition patterns, as estimated by phage ELISA

In the approach we used for estimating the conformational states induced by the respective compounds, each conformation is characterised by the unique combination of the peptides recognising it (Paige et al., 1999, Sumbayev et al., 2005). This combination is referred to as the peptide recognition pattern. For the experiments, we used an ELISA with streptavidin coated onto the solid phase, followed by a layer of biotinylated glutathione or biotinylated vitellogenin ERE; a layer of glutathione-S-transferase (GST)-ER α -LBD or full length human ER β , in combination with 1 μ M of various ligands; a layer of fusion phage displaying one of the peptides on its surface; and finally a layer of anti-phage antibody. Among the peptides, we chose 4 peptides, which allow the recognition of the specific ER α and ER β conformations induced by E2, oestriol (E3), OHT, and ICI 182,780, respectively: α/β I (SSNHQSSRLIELLSR); α/β II (SAPRATISHYLMGG);

α/β III (SSWDMHQFFWEGVSR); and α II (SSLTSRDFGSWYASR). The α/β I peptide contains the Leu-Xaa-Xaa-Leu motive characteristic for co-activators and binding of this peptide reflects the agonist conformation and the ability of a particular ligand to activate gene transcription. The other peptides bind at unrecognised sites in the LBD, and their binding is not obviously related to any specific transcriptional activity. In order to ensure that the peptides work according to expectancies (Paige et al., 1999), we demonstrated that α/β II recognises non-liganded receptors, α/β I recognises E2- and E3-complexed ER α and ER β , α/β III recognises OHT-complexed receptors, α I recognises ER α complexed with any ligand (Fig. 3).

As we wished to relate the ER conformations induced by a number of suspected environmental endocrine disruptors to the concept of "passive antagonism", we compared the peptide recognition pattern induced by these compounds to that induced by the passive antagonist prototype THC.

With ER α , THC induced a peptide recognition pattern very similar to that induced by E3, *i.e.*, α/β I+, α/β II+, α/β III-, and α II+ (Fig. 2 and Fig. 4). The same peptide recognition pattern of ER α was induced by diethylstilbestrol (DES), genistein, daidzein, bisphenol A (BPA), 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), methoxychlor, and 2,2'-dihydroxybenzophenone (DHBP) (Fig. 4). The recognition of ER α liganded with these compounds by α/β I is in agreement with the fact that they all, according to the literature, exhibit full agonistic behaviour in transactivation assays with ER α (Meyers et al., 1999; Sun et al., 1999; Andersen et al., 2002; Gaido et al., 1999). Within this group of ligands, the peptide recognition pattern induced into ER α H524A is different from that induced into ER α wt. A common trait for THC, DES, genistein, BPA, HPTE, methoxychlor, and DHBP, but not daidzein, is recognition of α/β III by the mutant, but not the wt (Fig. 4). In addition, the recognition by α/β I was lost with THC, DES, HPTE, methoxychlor, and DHBP, but not with genistein, daidzein, and BPA. One would therefore predict that these compounds stabilise the agonist conformation by an interaction with His524. In the cases of THC, DES, HPTE, and DHBP, this interaction could be a hydrogen bond between His524 and one of the hydroxyl groups, while methoxychlor would interact with His524 in another way, and genistein, daidzein, and BPA would maintain an active conformation in a completely different way. In conclusion, the peptide recognition pattern allows distinction of a group of compounds inducing an ER α conformation similar to that induced by E3, but still heterogeneous with respect to the exact molecular mechanism by which they maintain the active conformation.

With ER β , the peptide recognition pattern induced by THC was α/β I-, α/β II+, α/β III+ (Fig. 4). The lack of reaction with α/β I is in agreement with the fact that THC is an antagonist in transactivation assays with ER β (Shiau et al., 2002). The antagonists methoxychlor and HPTE induced a peptide recognition pattern identical to that induced by THC. The same pattern was induced by DHBP, but the transactivation properties of this compound is unknown. The peptide recognition pattern induced by the ER β agonists was α/β I+, α/β II+, α/β III-, a pattern similar to that induced by E3.

We previously reported that a group of polychlorinated pesticides with estrogenic agonistic behaviour induce a novel ER conformation, based on induction of a specific peptide recognition pattern, *i.e.*, α/β I+, α/β II-, α/β III+, α II+ with ER α and α/β I+, α/β II-, α/β III+ with ER β , and a lack of effect of introducing the H524A mutation into ER α . This peptide recognition pattern was referred to as the 2,4-dichlorodiphenyl-dichloroethylene (DDE) type or "polychlorinated pesticide" peptide recognition pattern (Sumbayev et al., 2005, Mukhopadhyay et al., 2005). This pattern is illustrated in Fig. 4 by DDT and DCBP. A comparison of the peptide recognition patterns induced by DDT and DCBP on one side to that induced by HPTE and DHBP on the other allows evaluation of the effect on the receptor conformation of having terminal hydroxyls groups versus terminal chlorine atoms. While the chlorinated compounds DDT is an agonist with both ER α and ER β , the hydroxylated compound HPTE is an agonist with ER α and an antagonist with ER β and have a quite different peptide recognition pattern, as described above. It is a "passive antagonist" in the sense of "antagonism without a side chain", and exhibits a peptide recognition pattern identical to that of THC. The functional activities of DCBP and DHBP are unknown, but interestingly, they have peptide recognition patterns identical to those of DDT and HPTE, respectively.

3.3. Ligand-dependent peptide recognition patterns, as estimated by surface plasmon resonance

To obtain more quantitative characteristics of peptide ER-LBD interactions, we performed investigations with surface plasmon resonance (SPR), using a BIACORE X instrument and a CM5 chip to immobilize α/β I, α/β II and α/β III peptides fused to the D1D2 phage coat protein. Five hundred nM receptor with or without ligand was injected. Association and then dissociation was registered. The association and dissociation rate constants were calculated from the association and dissociation phases of the binding

curves. The K_D values were calculated as the dissociation rate constants divided by the association rate constants. A summary of all results is given in Tables 2-4.

The peptide recognition patterns observed for the ligands were similar to those detected in the ELISA. Among the peptides with a measurable binding, there was little variation in the binding kinetics. There was a fast association (a k_1 of approximately $10^5 \text{ M}^{-1} \text{ s}^{-1}$) and slow dissociation (k_{-1} approximately $4 \times 10^{-4} \text{ s}^{-1}$) corresponding to K_D values in the nM range. We estimate that the K_D values, in the cases in which no binding could be detected, were more than 100 fold higher than that.

The data obtained with α/β I peptide are consistent with those reported for ER co-activators (Burakov et al., 2000, Wärnmark, et al., 2001).

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4. Discussion

In this communication, we describe experiments addressing agonistic and antagonistic properties of ER α and ER β ligands among hydroxylated and chlorinated biphenyls suspected of being environmental endocrine disruptors. In particular, we address the question whether some of these compounds can be classified as "passive antagonists", *i.e.*, antagonists which do not possess a side chain and thus act differently from the classical antagonists which by their side chain push α -helix 12 in a position incompatible with coactivator binding. We have done so by comparing the induced ER α and ER β peptide recognition patterns to that induced by THC, with special reference to the possibility of using ER α and ER β peptide recognition pattern in biosensors. For the receptor-peptide binding analyses, we used phage ELISA and surface plasmon resonance. On the phage particles used for the ELISAs, each peptide is displayed in several copies on the phage particles. There is therefore no simple relationship between the phage binding observed and the affinity of the peptides to the ERs. We therefore also measured the K_D values by the use of SPR analysis with a BIACORE instrument and could confirm the results obtained in the more semi-quantitative way by the phage ELISAs.

The phenomenon of "passive antagonism" was first described in the case of THC, which is a full antagonist in transactivation assays with ER β , but a full agonist with ER α (Shiau et al., 2002, Meyers et al., 1999; Sun et al., 1999). The X-ray crystal structure analysis of the THC-ER β LBD complex showed that by positioning certain binding pocket residues in non-productive conformations, the binding of THC to ER β disfavors the agonist-bound conformation of α -helix 12 and shifts the equilibrium towards the inactive conformation (Shiau et al., 2002). Accordingly, we found that the THC-ER β LBD complex does not bind to the $\alpha\beta$ I peptide, characteristic for classical agonistic behaviour, but induces a characteristic recognition of both $\alpha\beta$ II and $\alpha\beta$ III. This peptide recognition pattern is clearly different from that induced by the classical partial and complete "side chain" antagonists OHT and ICI 182,780. Among the other ER β antagonists tested, methoxychlor and HPTE gave a peptide recognition pattern identical to that of THC and therefore seem to stabilise an antagonistic conformation of the ER β -LBD in a way identical to THC. This peptide recognition pattern is shared by the closely related compound DHBP, with unknown transactivation activity. In agreement with the fact that they do not possess a side chain, methoxychlor, HPTE, and DHBP thus seem to induce "antagonism without a side chain" similarly to THC.

X-ray crystal structure analysis showed that α -helix 12 adopts very similar orientations in the THC-ER β LBD complex and the genistein-ER β LBD complex (Shiau et al., 2002). Based on the position of α -helix 12, the ER β LBD in the conformations stabilised by either THC and genistein should be incapable of interacting with co-activators, and hence, transcriptionally silent. Nevertheless, genistein and THC clearly show different activities on ER β in mammalian cells: Genistein is a partial agonist and THC is a pure antagonist (Pike et al., 1999; Shiau et al., 2002). The α/β I binding pattern is in excellent agreement with the latter observation, as the genistein-ER β complex recognises the α/β I peptide while the THC- ER β complex does not (see also Shiau et al., 2002). But how can the data from the X-ray crystal structure data be reconciled with the different activities of these compounds in transcriptional assays and the peptide binding assays? Shiau et al., 2002 proposed that in solution, α -helix 12 is in an equilibrium between the inactive conformations observed in the THC- and genistein-ER β LBD structure in the crystal and the active agonist-bound conformation. Ligands affect LBD transcriptional activity by shifting this equilibrium. Full agonists, like estradiol, shift α -helix 12 conformational equilibrium in favor of the active conformation and stimulate transcriptional activity by increasing the affinity of the LBD for co-activator. Because partial agonists, such as genistein, are incapable of shifting the equilibrium in favour of the active conformation to the same extent as full agonists, they are able only to incrementally increase the affinity of the receptor for coactivator and, hence, activate transcription less efficiently than full agonists. Genistein binding should permit the ER β LBD to sample both inactive and active conformations of α -helix 12. The observation of the inactive conformation in the genistein-ER β LBD complex in crystals may result from the influence of the crystallization conditions on the equilibrium. In contrast, THC binding forces the receptor to sample mainly the inactive conformation, in agreement with the absence of transcriptional activity of the THC-ER β complex and the conformation of helix 12 observed in the crystal.

THC is a full agonist in trans-activation assays with ER α . Accordingly, the X-ray crystal structure analysis showed that the three-dimensional structure of the THC-ER α LBD complex is very similar to that induced by DES (Shiau et al., 2002). The peptide recognition pattern induced by THC was identical to that induced by E3 and DES both with ER α wt and ER α H524A. Our peptide binding data show the solution structure may be somewhat more complex than indicated by the crystal structure, as the peptide recognition pattern induced by E3 and DES is clearly different from that induced by E2.

We previously reported that a group of polychlorinated pesticides, including DDT, induces a novel ER conformational state, which is different from the known conformations induced by the classical ligand types, but with a peptide recognition pattern which is the sum of the peptide recognition patterns induced by E2 and OHT (Sumbayev et al., 2005). The most ready explanation of this observation is pesticide-complexed ERs are in equilibrium between states resembling the more stable conformations induced by E2 and OHT, respectively. Interestingly, we find here that displacement of the terminal chlorine atoms with hydroxyl groups, which is part of the natural metabolism of these pesticides, has drastic effects on the peptide recognition patterns of their complexes with the ERs. Thus, DDT is an agonist while HPTE is an antagonist with ER β , with a corresponding difference in peptide recognition pattern. An identical difference is observed in the peptide recognition pattern induced in ER β with DCBP and DHBP, respectively. With ER α , in which the chlorinated as well as the hydroxylated compounds are agonists, the peptide recognition pattern changes from the polychlorinated pesticide pattern to a pattern identical to that induced by THC and E3.

Besides measuring the peptide recognition patterns induced by the different ligands, we also measured the K_D values for their binding to ER α wt and ER α H524A. Interestingly, the mutation changed the K_D 1.3 - 5.1 fold for all the compounds for which the peptide recognition pattern was changed by the mutation. This observation is in agreement with the notion that the binding of these compounds and their stabilisation of the bound conformation depend on interaction with His524. In contrast, in the cases of DDT and DCBP, the mutation neither changed the K_D nor the peptide recognition pattern. The only exception to this pattern was daidzein, for which the mutation, for reasons unknown at the moment, changed the K_D , but not the peptide recognition pattern.

Conclusively, we have demonstrated that a class of ER β agonistic suspected environmental endocrine disruptors affect ER conformation in the same way as THC, i.e., they are antagonists without a side chain. We have demonstrated that the peptide recognition pattern, although not being directly interpretable in terms of exact structural information, does give unique information about ER solution conformations which is not necessarily obtained by X-ray crystal structure analysis and in more direct agreement with results from trans-activation assays. Indeed, the peptide recognition pattern is able to register conformational differences induced by minor changes in the chemical structure of the ligands. The determination of the peptide recognition pattern is fast and simple, in contrast to other possible methods like partial trypsin digestion (Goldstein et al., 2001). The conformation-specific ER α - and ER β -peptide binding is therefore suitable as a

basis for biosensors (Mukhopadhyay et al., 2005; Hansen et al., 2007) for classification of suspected environmental endocrine disruptors.

The transactivation activity of the various compounds is tightly correlated with the ability to induce recognition of the $\alpha\beta 1$ peptide, which mimicks the receptor binding of co-activators. However, the very large variability in peptide recognition indicates further functional differences between the various compounds. Such differences are not easily studied by transfection-based transactivation assays, as these may be influenced by the exact promoter used; cell specific variations in co-activator and co-repressor expression; restricted penetration of the compounds into the cells; the compounds being activated or inactivated by cellular metabolism; binding to serum proteins present in the cultures. Further understanding of the functional importance of small conformational differences induced by different compounds could probably be achieved by correlating peptide-receptor binding with binding of the receptors to a battery of co-activators and co-repressors.

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Figure legends

Fig. 1. Conformations of ER α and ER β LBD in complex with pure antagonist (E2, to the left); a selective estrogen receptor modulator (Ral, raloxifene, in the middle); a “passive” antagonist (THC, to the right). The figure shows positioning of the ligand (green) in the ligand-binding cavity as well as positioning of the α -helix 12 (red), in two different orientations for each receptor-ligand complex. The figures were constructed by the use of SWISSPDBVIEWER, using the pdb files 1ERE, 2J7X, 1ERR, 1QKN, 1L2I and 1L2J respectively.

Fig. 2. Examples of determination of K_D values for the binding of ligands to ER α wt and ER α H524A. Tritiated E2 (0.8 nM) and various concentrations of non-radioactive ligands (E2 (circles) or DDT (triangles)) were incubated for 2 h at 37°C with GST-ER α -LBD wt (closed symbols) or H524A (open symbols) (3 pmol per well) immobilised in microtiter wells coated with streptavidin and biotinylated GSH (100 pmol per well). After the incubation, aliquots of the supernatants were taken for liquid scintillation counting. Receptor-bound tritiated E2 was eluted and subjected to liquid scintillation counting. The K_D values for binding of the ligands to the receptor were by fitting the experimental data points to the equation:

$$[\text{bound E2}]/[\text{free E2}] = [\text{R}]_T / ([\text{free E2}] + K_{D, E2} + (K_{D, E2}/K_{D, \text{ligand}}) \cdot [\text{ligand}]) + C$$

in which $[\text{R}]_T$ is the total receptor concentration, $K_{D, E2}$ is the K_D value for binding of E2, $K_{D, \text{ligand}}$ is the K_D value for the ligand in question, $[\text{ligand}]$ is the concentration of the ligand, and C is a constant. The SigmaPlot software was used for the analyses. In the figure, the $[\text{bound E2}]/[\text{free E2}]$ -values were plotted as fraction of the maximum value attained in the absence of ligand, versus the logarithm of the ligand concentration.

Fig. 3. Peptide recognition patterns displayed by ER conformations induced by established classic natural and synthetic ligands. To the left, a schematic outline of the ligand-induced α -helix 12 positions. Peptide recognition patterns induced by the indicated ligands in ER α wt., ER α and ER β , as evaluated by phage ELISA. ELISA format I was used for ER β , while ELISA format II was used for ER α wt and ER α H524A.

Fig. 4. Peptide recognition pattern of ER α and ER β complexed with non-steroid pure agonists, passive antagonists, and polychlorinated biphenyls. The binding of the indicated peptides to GST-ER α -LBD wt (■),

GST-ER α -LBD-H524A (\square), and full length ER β in the presence of 1 μ M of the indicated ligands was estimated by phage ELISA. ELISA format I was used for ER β , while ELISA format II. The signals obtained with peptides α/β I and ER α in the presence of E2 were set to 100%, and the signals obtained with other peptide/ligand combinations expressed as a fraction thereof. Data are shown as means \pm S. D. of at least 5 individual experiments.

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Table 1. K_D values for the binding of ligands to ER α wt and ER α H524A. The K_D values were estimated from the ability of the ligands to compete tritiated E2 out of the receptor. The table shows mean and standard deviations for 3 independent determinations. (E2 – oestradiol, E3 – oestriol, OHT – 4-hydroxytamoxifen, THC – 5,11-cis-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol, DES – diethylstilbestrol, BPA – bisphenol, HPTE – 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, DHBP – 2,2'-dihydroxybenzophenone, DDT – 2,4-dichlorodiphenyl-dichloroethane, DCBP – 2,2'-dichlorobenzophenone).

Ligand	Receptor		
	ER α -LBD wt K_D , nM	ER α -LBD H524 K_D , nM	Ratio K_D (H524)/ K_D (wt)
E2	1.3 \pm 0.2	5.4 \pm 0.8 ^a	4.1
E3	2.2 \pm 0.3	12 \pm 2 ^a	5.5
OHT	1.1 \pm 0.2	5.6 \pm 0.4 ^a	5.1
THC	14 \pm 1	26 \pm 3 ^a	1.9
DES	19 \pm 2	34 \pm 2 ^a	1.9
Genistein	26 \pm 3	69 \pm 4 ^a	2.7
Daidzein	93 \pm 9	144 \pm 18 ^a	1.5
BPA	42 \pm 6	162 \pm 11 ^a	3.8
HPTE	30 \pm 3	63 \pm 8 ^a	2.1
Methoxychlor	350 \pm 21	456 \pm 35 ^a	1.3
DHBP	69 \pm 9	135 \pm 13 ^a	2.0
DDT	75 \pm 6	65 \pm 6	0.9
DCBP	125 \pm 14	116 \pm 17	0.9

^aSignificantly different from K_D value for wt ($p < 0.01$).

Table 2. Rate constants and K_D values for the interaction between conformation-specific peptide α/β I and ERs, as estimated by SPR. The indicated peptides, in fusion with D1D2, were immobilised on a BIACORE CM5 chip. The indicated receptor preparations were passed over the chip, and the rate constants for association and dissociation (k_1 and k_{-1}) and the dissociation equilibrium binding constant (K_D) were determined as described in "Experimental procedures". "No binding" indicates that no binding could be recorded with 500 nM receptor.

Ligand	Receptor								
	ER α -LBD wt			ER α -LBD H524			ER β		
	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	K_D (M)	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	K_D (M)	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	K_D (M)
No ligand	No binding			No binding			No binding		
E2	1.3×10^5	3.3×10^{-4}	2.5×10^{-9}	1.0×10^5	4.3×10^{-4}	4.2×10^{-9}	1.3×10^5	4.0×10^{-4}	3.0×10^{-9}
E3	1.3×10^5	3.4×10^{-4}	2.7×10^{-9}	1.0×10^5	4.8×10^{-4}	4.7×10^{-9}	1.4×10^5	3.5×10^{-4}	2.5×10^{-9}
OHT	No binding			1.1×10^5	11×10^{-4}	10.3×10^{-9}	No binding		
THC	1.2×10^5	3.3×10^{-4}	2.8×10^{-9}	No binding			No binding		
HPTE	1.1×10^5	4.0×10^{-4}	3.8×10^{-9}	No binding			No binding		
DDT	1.1×10^5	4.5×10^{-4}	4.0×10^{-9}	1.0×10^5	4.1×10^{-4}	4.0×10^{-9}	1.0×10^5	4.6×10^{-4}	4.5×10^{-9}

Table 3. Rate constants and K_D values for the interaction between conformation-specific peptide α/β II and ERs, as estimated by SPR. The indicated peptides, in fusion with D1D2, were immobilised on a BIACORE CM5 chip. The indicated receptor preparations were passed over the chip, and the rate constants for association and dissociation (k_1 and k_{-1}) and the dissociation equilibrium binding constant (K_D) were determined as described in "Experimental procedures". "No binding" indicates that no binding could be recorded with 500 nM receptor.

Ligand	Receptor								
	ER α -LBD wt			ER α -LBD H524			ER β		
	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	K_D (M)	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	K_D (M)	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})	K_D (M)
No ligand	0.72×10^5	0.79×10^{-4}	1.1×10^{-9}	0.71×10^5	0.81×10^{-4}	1.2×10^{-9}	0.72×10^5	0.80×10^{-4}	1.1×10^{-9}
E2	No binding			No binding			No binding		
E3	1.4×10^5	5.4×10^{-4}	4.0×10^{-9}	1.8×10^5	5.9×10^{-4}	3.3×10^{-9}	1.2×10^5	5.1×10^{-4}	4.4×10^{-9}
OHT	No binding			No binding			No binding		
THC	1.1×10^5	4.8×10^{-4}	4.6×10^{-9}	1.0×10^5	6.8×10^{-4}	6.6×10^{-9}	1.0×10^5	5.3×10^{-4}	5.2×10^{-9}
HPTE	0.93×10^5	5.9×10^{-4}	6.4×10^{-9}	0.79×10^5	5.3×10^{-4}	6.7×10^{-9}	0.89×10^5	5.4×10^{-4}	6.1×10^{-9}
DDT	No binding			No binding			No binding		

Table 4. Rate constants and K_D values for the interaction between conformation-specific peptide α/β III and ERs, as estimated by SPR. The indicated peptides, in fusion with D1D2, were immobilised on a BIACORE CM5 chip. The indicated receptor preparations were passed over the chip, and the rate constants for association and dissociation (k_1 and k_{-1}) and the dissociation equilibrium binding constant (K_D) were determined as described in "Experimental procedures". "No binding" indicates that no binding could be recorded with 500 nM receptor.

Ligand	Receptor								
	ER α -LBD wt			ER α -LBD H524			ER β		
	k_1 (M $^{-1}$ s $^{-1}$)	k_{-1} (s $^{-1}$)	K_D (M)	k_1 (M $^{-1}$ s $^{-1}$)	k_{-1} (s $^{-1}$)	K_D (M)	k_1 (M $^{-1}$ s $^{-1}$)	k_{-1} (s $^{-1}$)	K_D (M)
No ligand	No binding			No binding			No binding		
E2	No binding			0.84×10^5	5.0×10^{-4}	5.9×10^{-9}	No binding		
E3	No binding			0.80×10^5	9.6×10^{-4}	12×10^{-9}	No binding		
OHT	3.0×10^5	7.4×10^{-4}	2.5×10^{-9}	2.4×10^5	9.2×10^{-4}	3.9×10^{-9}	2.7×10^5	6.9×10^{-4}	2.6×10^{-9}
THC	No binding			0.87×10^5	8.8×10^{-4}	10×10^{-9}	2.6×10^5	5.9×10^{-4}	2.3×10^{-9}
HPTE	No binding			2.0×10^5	8.1×10^{-4}	4.0×10^{-9}	2.6×10^5	11×10^{-4}	4.1×10^{-9}
DDT	1.9×10^5	8.1×10^{-4}	4.3×10^{-9}	2.7×10^5	11×10^{-4}	4.1×10^{-9}	1.8×10^5	7.3×10^{-4}	4.0×10^{-9}







