Interference between Progesterone and Dioxin Signal Transduction Pathways

DIFFERENT MECHANISMS ARE INVOLVED IN REPRESSION BY THE PROGESTERONE RECEPTOR A AND B ISOFORMS*

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Interactions between transcription factors are an important means of regulating gene transcription, leading to modifications in the pattern of gene expression and cell fate. In this study, we report that the progesterone receptor (PR) can strongly interfere with transactivation mediated by the arylhydrocarbon receptor (AhR) in T47D breast cancer cells. This interference was not only demonstrated by induction of a transfected dioxin-responsive reporter plasmid but also on the AhR-mediated up-regulation of the endogenous cytochrome P450-1A1 activity. The interference was not mutual, as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent activator of the AhR, did not inhibit progesterin-induced promoter activity. When the isoforms of the human PR, hPR-A and hPR-B, were expressed separately in HepG-2 hepatocarcinoma cells, both negatively interfered with the AhR signaling, indicating that the effect is not restricted to T47D cells. In addition, results obtained from studies with both antiprogestins and mutant receptors indicate differences in the underlying molecular mechanisms of repression for both PR isoforms. The suppression by hPR-A does not require additional gene expression or a full transcriptional competent conformation of the receptor. For the repressive effects of hPR-B, however, additional gene expression seems to be involved, as only the agonist-bound, wild-type hPR-B could clearly repress the TCDD-induced response. In conclusion, these studies highlight different mechanisms of repression for the progesterone receptor isoforms on the AhR-mediated trans-activation and underscore the importance of interactions between transcription factors of different families in the regulation of gene transcription.

The progesterone receptor (PR) belongs to a large superfamily of conserved nuclear proteins, including receptors for steroid, retinoid, and thyroid hormones (1). These proteins, which share common functional domains responsible for properties such as ligand binding, dimerization, DNA binding, and trans-activation, act as ligand-activated transcription factors in the tissues where they are expressed. These receptors recognize and bind specific DNA regulatory sequences, so-called hormone-responsive elements, finally resulting in the activation of gene transcription. The PR, which belongs to a subfamily including the androgen and glucocorticoid receptors, is unique among members of the steroid receptor family in that it occurs in target tissues as two distinct isoforms, PR-A and PR-B (2). The human PR-B contains an additional 164 amino acids at its N terminus that are absent in the hPR-A. Both isoforms of the hPR can mediate progesterin-activated gene transcription, but they do so in a promoter- and cell-specific manner (3–5).

Besides their effects as activators of gene transcription, the PR can influence gene transcription by an interplay with other transcription factors, most frequently resulting in an inhibition of gene transcription. Several mechanisms for this so-called cross-talk have been described. In transcriptional interference experiments, the presence of the PR can suppress the activity of other related trans-activating factors by the sequestration of putative bridging factors (6, 7) or by occupying a surface required to mediate or receive trans-acting signals (5, 8), a phenomenon referred to as surface saturation (9). Furthermore, transcriptional interference can occur due to a competition between receptors for a common DNA element in the target promoter (5). Finally, the PR can influence gene transcription through direct interactions with the transcription factors from distinct families, AP-1 (10) and NF-κB (11).

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and several structurally related halogenated aromatic compounds disrupt diverse endocrine-responsive pathways, including those involving steroid hormones (reviewed in Refs. 12–14). These compounds act through the activation of a transcription factor, the aryl hydrocarbon receptor (AhR), which subsequently heterodimerizes with the AhR nuclear translocator protein (Arnt) and binds to a so-called xenobiotic responsive element (15). The AhR and Arnt belong to the basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family of transcription factors (16) and have a similar overall structure, which is different from the ligand-activable steroid hormone receptors. Upon binding to xenobiotic responsive elements, the heterodimeric AhR-Arnt complex activates transcription of genes, encoding especially cytochrome P450s (e.g., 1A1), that are involved in the oxidative metabolism of these xenobiotic compounds.

Several mechanisms have been postulated to explain the AhR-mediated interference with steroid hormone-regulated responses, including altered hormone synthesis (17), enhanced...
Different Mechanisms of Repression for PR Isoforms

HepG-2 cells were cultured in 24-well tissue culture plates at a density of 250,000 cells per well with 5% fetal calf serum, which was heat-inactivated and treated with serum. Australia). Cells were maintained in a 1:1 ratio of Dulbecco's modified Eagle's medium (DMEM) and HEPES-buffered salts. For transfection studies, cells were grown in phenol red-free DMEM and transfected by the calcium phosphate coprecipitation method with 5% fetal calf serum, which was heat-inactivated and treated with dextran-coated charcoal to remove steroidogenic components (25). The expression plasmids for the DNA-binding deficient PR isoforms (hPR-Acys and hPR-Bcs; Cys located at the base of the first zinc cluster mutated to Ala (23)) and the PR isoforms with altered DNA-binding specificity (hPR-Aspec and hPR-Bspec; recognizing the estrogen-responsive element (23)) were kindly provided by Dr. K. Horwitz (Denver, CO). The T47D breast tumor cells and HepG-2 hepatocarcinoma cells. In addition, we studied the endogenous TCDD-induced cytochrome P450-1A1 activity in T47D cells. We demonstrate that both PR isoforms can act as potent repressors of the AhR activity and that agonist- and antagonist-occupied PRs differentially modulate AhR function. Our studies, which address mechanistic aspects of the repressive effects of PR on the AhR signaling, add a new aspect to the expanding network of interactions between transcription factors of different families in the regulation of gene transcription. In addition, the present data suggest that progesterins may protect cells against the detrimental effects of TCDD and other halogenated compounds.

**Experimental Procedures**

**Materials**—TCDD was kindly provided by Dr. M. van den Berg (RITOX, Utrecht, The Netherlands). The progestin Org2058 was a gift from Organon (Oss, The Netherlands), RU486 was from Roussel Uclaf (Romainville, France), and ZK98229 was from Schering (Berlin, Germany). All other steroids were purchased from Sigma.

**Expression and Reporter Plasmids**—Preparation of the progesterone-responsive reporter plasmid PRE2-tk-LUC and the expression plasmids for hPR-A and hPR-B were described previously (11). The expression plasmids for the DNA-binding deficient PR isoforms (hPR-Acys and hPR-Bcs; Cys located at the base of the first zinc cluster mutated to Ala (23)) and the PR isoforms with altered DNA-binding specificity (hPR-Aspec and hPR-Bspec; recognizing the estrogen-responsive element (23)) were kindly provided by Dr. K. Horwitz (Denver, CO). The TCDD-responsive reporter plasmid GUD-LUC 1.1 (24), containing a 480-base pair fragment of the mouse cytochrome P450-1A1 promoter including four xenobiotic responsive elements, was a gift from Dr. M. S. Denison (Davis, CA). The RARE-tk-LUC reporter plasmid was prepared by cloning an oligonucleotide containing the retinoic acid-responsive element (RARE) as found in the RARβ promoter in the HindIII-digested tk-LUC plasmid.

**Cell Culture and Transfections**—Human HepG-2 cells were purchased from the American Type Culture Collection (Rockville, MD), and T47D breast tumor cells were purchased from Dr. R. L. Sutherland (Sydney, Australia). Cells were maintained in a 1:1 ratio of Dulbecco's modified Eagle's medium/Ham's F12 medium supplemented with 7.5% fetal calf serum.

For transfection studies, cells were grown in phenol red-free Dulbecco's modified Eagle's medium/Ham's F12 medium supplemented with 5% fetal calf serum, which was heat-inactivated and treated with dextran-coated charcoal to remove steroidogenic components (25). HepG-2 cells were cultured in 24-well tissue culture plates at a density of 3 × 10^4/cm^2 and transfected by the calcium phosphate coprecipitation method with 400 μg of reporter plasmid, 200 ng of expression plasmid, and 600 ng of PDM-LacZ control plasmid. pBluescript SK+ plasmid was added to obtain a total of 1.5 μg of DNA per well.

**Measurement of EROD Activity**—Cell lysates of T47D cells were prepared, upon incubation with the experimental media, by the addition of 250 μl of H2O and were analyzed for EROD activity essentially as described previously (26). In brief, 50 μl of Tris-sucrose buffer (pH 8.0) with 40 μM dexamethasone was added to 20 μl of lysate, followed by 25 μl of 10 μM 7-ethoxycoumarin. The reaction was started by the addition of 25 μl of 1 mM NADPH and incubated for 1 h at 37 °C. Formation of resorufin was measured spectrofluorometrically at 530-nm excitation and 590-nm emission wavelengths.

**RESULTS**

Effect of Progestosterone Receptor Ligands on TCDD Response in T47D Cells—Interference of the progestosterone and dioxin signal transduction pathways was tested in T47D human breast cancer cells in which the PR, AhR, and its dimerization partner Arnt are endogenously expressed. Transfection of the T47D cells with the GUD-LUC 1.1 reporter plasmid, containing four xenobiotic responsive elements, resulted in a clear induction of luciferase activity upon the addition of TCDD (Fig. 1A). However, simultaneous addition of TCDD with the synthetic progestin Org2058 resulted in a dramatic inhibition of the TCDD response (Fig. 1A). To confirm the down-regulation of the TCDD response by progesterone in a more natural context, we also studied the AhR-mediated induction of endogenous cytochrome P450 (CYP)-1A1 in these cells. The EROD activity, a CYP1A1-mediated catalytic activity, is enhanced upon the addition of TCDD and was inhibited dose dependently by Org2058 until maximal repression was reached at 10 μM Org2058 (Fig. 2).

To define more precisely the mechanism of this PR-mediated repression, the TCDD response was examined in the presence of two different antiprogestins. The first compound, RU486, promotes the association of PR with DNA, whereas the second, ZK98299, interferes with DNA binding, presumably by interfering with dimerization of the receptor (23, 27). Surprisingly, both antiprogestins RU38486 and ZK98299 down-modulate the activity of the AhR-Arnt complex in T47D cells to the same extent, although the observed repression was not as strong as in the presence of the agonist Org2058 (Fig. 1A).

Next, we performed the reciprocal experiments to examine the effects of the addition of TCDD on the PR response. In contrast to the progestin-mediated down-regulation of the TCDD response, TCDD did not inhibit the progestin-induced activity on the PRE2-tk promoter (Fig. 1B). However, the low agonistic activity of RU486, as found on the PRE2-tk-LUC reporter, was inhibited upon the addition of TCDD, suggesting that TCDD can interfere with some aspects of the PR response.

The down-regulation of the TCDD response was not due to a general repression of transcriptional activity by the PR. First, the hormone-dependent trans-activation by the retinoic acid receptor, assayed in T47D cells upon transfection of the retinoic acid-responsive reporter plasmid RARE-tk-LUC, was not affected by the presence of Org2058 (Fig. 1C). In addition, we neither observed effects upon the transcriptional activity of the SV40 promoter (assayed as a fusion to β-galactosidase, data not shown).

From these studies, we conclude that, at least in T47D cells, hPR showed strong interference with the transactivation ability of the AhR-Arnt complex in a ligand-dependent manner.

**Antiprogestin-bound Progesterone Receptor Isoforms Differentially Inhibit the TCDD Response**—The biological response to progestins is mediated by two isoforms of the human PR (hPR-A and hPR-B), which are both present in T47D cells (2) and have differential effects in gene activation and repression (3–5). Therefore, the mechanism of inhibition of the TCDD response by the progestins and antiprogestins was investigated in HepG-2 human hepatocarcinoma cells, which express neither hPR-A nor hPR-B. In these cells, the TCDD reporter plasmid GUD-LUC 1.1 was activated in a ligand-dependent manner by the endogenous AhR-Arnt complex (Fig. 3), and
addition of either progestins or antiprogestins had no effect (data not shown). However, upon cotransfection of the expression plasmid encoding either hPR-A or hPR-B, Org2058 again repressed the TCDD-mediated response, being slightly more active with hPR-B (compare Fig. 3, A and B). This PR-mediated repression of the TCDD response in HepG-2 cells indicated that the effect is not restricted to T47D cells and might be of more general importance.

Next, the ability of the antiprogestins RU486 and ZK98229 as potential transcriptional repressors of the TCDD response was evaluated in these cells. In the presence of hPR-A, both RU486 and ZK98229 efficiently repressed the TCDD response (Fig. 3A). Interestingly, this transcriptional repression was even stronger than in the presence of the agonist Org2058. The results of this analysis indicated that hPR-A could function as a repressor in the presence of both agonists and antagonists. Furthermore, as the antiprogestin ZK98229 inhibits DNA binding of the receptor (27), interaction with the cognate DNA seems not to be required for the hPR-A-mediated repression.

In contrast, the ZK98229-bound hPR-B was unable to repress the transcriptional activity of the AhR-Arnt complex (Fig. 3B), suggesting that the hPR-B could only suppress the TCDD response in a transcriptionally active and/or DNA-bound form. This suggestion could be substantiated with the compound RU486, which induces DNA binding of the hPR (28) and shows partial agonistic activity in a cell- and promoter-dependent context (Fig. 1; Ref. 29). Upon cotransfection of hPR-B, addition of this latter compound resulted in a partial repression of the TCDD response only.

In summary, these results indicate that antiprogestin-mediated repression of the TCDD response is differentially exerted by the ligand-bound hPR-A and hPR-B and therefore open the possibility of different mechanisms behind the repressive effects of both PR isoforms.

**DNA Binding Mutants of hPR-A, but Not hPR-B, Are Effec-

**FIG. 1.** A and B, transcriptional interference properties of TCDD and (anti)progestins. T47D cells were transfected with either the TCDD-responsive reporter GUD-LUC 1.1 (A) or the progestin-responsive reporter PRE-tk-LUC (B) and an internal control plasmid encoding β-galactosidase. After transfection, cells were incubated in the presence of Org2058 (10⁻⁷ M), RU486 (10⁻⁶ M), and ZK98229 (10⁻⁶ M) in the absence (hatched bars) or presence (black bars) of TCDD (10⁻³ M) for 24 h. Upon incubation, cell lysates were assayed for luciferase and β-galactosidase. Fold induction was determined after correction for transfection efficiency and represents the mean ± S.E. of at least three independent experiments, each performed in triplicate. C, effect of the progestin Org2058 on the retinoic acid response. T47D cells were transfected with the RARE-tk-LUC reporter plasmid and an internal control plasmid. Cells were treated with all-trans retinoic acid (RA, 10⁻⁶ M), Org2058 (10⁻⁷ M), or both hormones in combination as indicated. The data represent the mean ± S.E. of three independent experiments, each performed in duplicate.

**Fig. 2.** Inhibition of TCDD-mediated induction of EROD activity in T47D cells by progestins. T47D cells were incubated with TCDD (10⁻⁹ M) and the indicated concentrations of the synthetic progestin Org2058 (10⁻¹²-10⁻⁷ M, indicated as −12 to −8). Catalytic activity of the endogenous TCDD-inducible cytochrome P450-1A1 was determined in an EROD assay as described under “Experimental Procedures.” Results represent the mean ± S.E. of duplicate determinations.
To further examine the potential different mechanisms behind the repressive effects of the PR isoforms more directly, we used two sets of mutants that were altered in their DNA binding domains. In hPR-Aspec and hPR-Bspec, the specificity of the DNA binding domain was altered such that, in gel shift experiments, an estrogen-responsive element (ERE) is recognized (23). The transcriptional activity of these mutant receptors was tested in HepG-2 cells. As shown in Fig. 4A, the mutant hPR-Bspec was transcriptionally active on an ERE3-tata-LUC reporter in an Org2058-dependent manner. In contrast, no Org2058-induced activation of the same reporter was observed by hPR-Aspec. When hPR-Bspec was cotransfected with the hPR-Aspec, hPR-Aspec functions as a repressor of the hPR-Bspec activity (Fig. 4A). This repressive effect of the PR-Aspec isoform could also be observed with both wild-type receptor isoforms in which hPR-A can suppress hPR-B activation (Fig. 5B) and is in agreement with observations made previously (4, 5, 30). The inhibition of the transcriptionally inactive hPR-A on the hPR-B-induced promoter activity may result from the formation of non-functional hPR-AhPR-B heterodimers. In the other set of mutants, termed hPR-Acys and hPR-Bcys, the amino acid residue cysteine located at the base of the first zinc cluster is replaced by an alanine residue. These mutants were not capable of binding DNA in vitro, as demonstrated by gel shift experiments, and were transcriptionally inactive on a PRE2-tk-LUC reporter plasmid (Refs. 23, 30, and data not shown).

We then tested the effects of these DNA binding mutant receptors on the endogenous TCDD response in HepG-2 cells. This was accomplished by transfecting the expression vector in combination with the TCDD-responsive reporter plasmid. This analysis revealed that both the progestin-bound hPR-Acys and hPR-Aspec, the amino acid residue cysteine located at the base of the first zinc cluster is replaced by an alanine residue. These mutants were not capable of binding DNA in vitro, as demonstrated by gel shift experiments, and were transcriptionally inactive on a PRE2-tk-LUC reporter plasmid (Refs. 23, 30, and data not shown).

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Different Mechanisms of Repression for PR Isoforms

The present study demonstrates a ligand-induced, unilateral transcriptional interference of the progesterone-responsive gene, whereas the hPR-A interacts through a mechanism not involving transcriptional activation.

DISCUSSION

The precise in vivo functions of hPR-A and hPR-B have not been defined yet. Previously, it has been demonstrated that the transcriptional activities of both isoforms are different and are dependent upon cell and promoter context (4, 5, 29). In general, the hPR-A isoform is transcriptionally less active and, as demonstrated by us and others (4, 5), functions as a dominant inhibitor of hPR-B function. In addition, hPR-A inhibits the transcriptional activity of various steroid hormone receptors (4, 31, 32). Furthermore, our present findings on the ability of hPR-A to regulate the activity of the AhR and its heterodimeric partner Arnt suggest that hPR-A can also influence the activity of transcription factors belonging to different families. In fact, these results enlarge the suggested central role for hPR-A in regulating transcriptional activities in those cells in which it is expressed (5).

Our findings are consistent with those of other reports demonstrating that liganded PR can have potent inhibitory effects on other transcriptional activators (4, 5, 11, 31, 32). Our results do differ from most of these studies in that the repression in our system was not limited to one isoform of the hPR but was observed with both isoforms. The exact mechanism of hPR-mediated inhibition of AhR action is currently unknown. Several mechanisms can be proposed to explain the repressive effect of hPR on the AhR-mediated response including 1) sequestration of general and/or specific transcription factors, 2) occupation of a surface required to mediate or receive trans-acting signals, 3) direct interaction between both factors, 4) the induction of a specific protein that interferes with the dioxin signaling pathway; also, repression can occur due to a competition between receptors for a common DNA element in the target promoter. This last mechanism, however, can be excluded as both factors recognize specific but distinct DNA regulatory elements. According to us, sequestration of specific and/or general transcription factors is a less likely mechanism for repression. An important aspect in this mechanism of repression is the mutual effect due to competition for such a factor, as demonstrated in transcriptional interference or squelching experiments within the nuclear hormone receptor superfamily (6, 7). Transfection of expression plasmids encoding AhR and Arnt in T47D cells did neither release the repressive effects of hPR-B nor induce repression of the progesterin-induced promoter activity, clearly demonstrating the unilaterality of the interference. In analogy, a direct interaction between both transcription factors, as described for the interaction of PR with both NF-κB (11) and AP-1 (10), seems not to be a major mechanism. However, the repressive effect of TCD on the partial agonistic activity of RU486 on the PR-responsive promoter still indicates some kind of mutual interference.

The fact that ZK98299-occupied receptors and PRE specific mutant-A receptors were able to repress transcription of the TCD-occupied AhR-Arnt complex leads to the conclusion that suppression of the AhR-mediated transcription by the hPR-A also proceeds without direct binding of hPR-A to DNA. In addition with the inability of hPR-A to induce transcription in HepG-2 cells, these results indicate that the repressive effect of hPR-A most likely occurs by occupation of a surface required to mediate the trans-acting signals of the AhR-Arnt complex, a mechanism previously proposed by others for its interference with other steroid hormone receptors (5, 8).

In contrast to hPR-A, hPR-B only represses transcription of the AhR-Arnt complex in its agonist-bound and PRE-recognizing form. These clear differences between both isoforms, which most probably originate from the additional 164 N-terminal amino acid residues present in the hPR-B isoform, do not completely exclude that occupation of a surface required to

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2 C. W. Kuil, unpublished data.
mediate the trans-acting signals of the AhR-Arnt complex is the major molecular mechanism of repression for both isoforms. However, together with the observation that the agonist-bound hPR-B is transcriptionally active, our results suggest that repression of gene activity by the hPR-B is acquired through the induction of (a) protein(s) that interferes with the dioxin signaling pathway. Potential candidates could be proteins from the expanding basic helix-loop-helix/Per-Arnt-Sim family, such as PER and HIF1α, which dramatically down-regulate the TCDD-mediated regulation of a reporter construct (33, 34). Additional studies, however, are necessary to pinpoint the exact mechanism(s) of repression of the PR isoforms on the dioxin signal transduction pathway.

Most, if not all, of the toxic effects of TCDD are the result of interaction with the AhR protein. This interaction clearly results in the induction of several metabolizing enzymes, such as cytochrome P450s. The repressive effect of the progestin Org2058 on the transfected reporter system was confirmed on the endogenously TCDD-inducible CYP-1A1 activity in T47D cells. In analogy, both progesterone and cortisol, a ligand for the glucocorticoid receptor, have been shown to suppress Cyp-1A1 and Cyp-1B1 mRNA levels in cultured rat mammary cells (35). Together, these findings implicate that in those tissues in which both receptor systems are present, such as breast and endometrium, progestins may protect against the detrimental effects of TCDD and other halogenated compounds, causing an altered synthesis or an increased metabolism of hormones. Several in vivo studies suggested that TCDD is involved in the pathogenesis of endometriosis (36–38), a disease in which endometrial tissue grows outside of the uterus. Progestins, but also antiprogestins, are frequently used in the treatment of this chronic disease. In accordance with the present data, part of the benefits of the treatment with PR ligands may include repression of the AhR response induced by environmental contaminants, such as TCDD.

In summary, the ability of hPR to regulate the activity of the AhR underscores the potential importance of interactions between transcription factors of different families in the regulation of gene transcription. Identification of the targets for both isoforms of the hPR, involved in the down-regulation of the AhR response, and the elucidation of their precise mechanism of action will facilitate additional insights in the mechanisms of TCDD toxicity and the potential beneficial effects that the ligands, either agonist or antagonist, of the PR could have.

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