Agonistic and Antagonistic Effects of α-Naphthoflavone on Dioxin Receptor Function

ROLE OF THE BASIC REGION HELIX-LOOP-Helix DIOXIN RECEPTOR PARTNER FACTOR Arnt*

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The dioxin receptor is a ligand-dependent transcription factor that binds to target DNA sequences (xenobiotic responsive elements, XREs) following ligand-dependent activation with its partner factor, Arnt (aryl hydrocarbon receptor nuclear translocator). Both factors contain an N-terminal basic region helix-loop-helix motif mediating dimerization and subsequent DNA binding. In this study we investigate the possible role of Arnt in agonistic and antagonistic effects of the dioxin receptor ligand α-naphthoflavone (ANF). Using specific antisera for the ligand binding dioxin receptor and Arnt, respectively, we show that exposure of the dioxin receptor to ANF in vitro induced recruitment of Arnt, thus stimulating binding of the heteromeric complex to XRE. In transient transfection assays, ANF at high concentrations stimulated expression of an XRE-driven reporter gene. This agonistic effect of ANF is, therefore, most likely attributable to ANF stimulation of dioxin receptor-Arnt heterodimerization and subsequent binding of the complex to XRE. Using a minimal XRE-driven reporter gene construct, we could further confirm earlier studies showing that ANF antagonizes the effect of a dioxin receptor agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin. Next we employed chimeric receptor constructs containing amino acids 1-500 of the human aryl hydrocarbon receptor fused to dioxin receptor fragments lacking the very N-terminal basic region helix-loop-helix dimerization and DNA binding motif. These chimeric receptor constructs show dioxin responsiveness upon transient transfection into mutant Arnt-deficient hepatoma cells and are, thus, functionally uncoupled from Arnt. Importantly, dioxin-dependent activation of the chimeric receptors was inhibited in the presence of ANF, demonstrating that dimerization of dioxin receptor with Arnt was not necessary for manifestation of the antagonistic effect of ANF. Rather, dioxin receptor sequences, which confer dioxin regulation upon a heterologous DNA binding and transactivating domain, also mediated the antagonistic effects of ANF.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (dioxin, TCDD)† induces transcription of a battery of target genes including the cytochrome P450IA1 gene via the intracellular basic helix-loop-helix (bHLH) dioxin receptor, also termed aryl hydrocarbon receptor (Emn et al., 1992; Burbach et al., 1992). Upon exposure to dioxin, the receptor acquires an increased affinity for target regulatory DNA sequences (xenobiotic responsive elements, XREs) that confer dioxin regulation on target promoters. Activation of the receptor to an XRE binding form is accompanied by its apparent translocation from the cytoplasm to the nucleus of target cells (reviewed by Poellinger et al. (1992)). The receptor activation pathway is not fully understood but is clearly a multistep process.

In nonstimulated cells the receptor is recovered as a ~300-kDa complex containing the ligand binding dioxin receptor and the molecular chaperon hsp90 (90-kDa heat shock protein) (Denis et al., 1988; Perdew, 1988). There may be other components of this large receptor form (Perdew, 1992), but these have so far not been identified. Binding of ligand induces release of hsp90 from the ~100-kDa receptor (Wilhelmssson et al., 1990) and dimerization (Whitelaw et al., 1993b; Matsushita et al., 1993; Probst et al., 1993) with the structurally related bHLH factor Arnt (Hoffman et al., 1991). The nuclear receptor-Arnt heterodimer shows strong XRE binding activity (Reyes et al., 1992; Dolwick et al., 1993; Whitelaw et al., 1993b) and, through an unclear mechanism, alters the transcription rate of target genes (for a review, see Fujii-Kuriyama et al. (1992)).

Among dioxin receptor ligands there seems to be a good correlation between the affinity of a given ligand, its in vitro activating capacity (measured as induction of XRE binding activity of the receptor), and its potency to induce transcription of the cytochrome P450IA1 gene in vivo (Cuthill et al., 1991). α-Naphthoflavone (ANF) binds to the dioxin receptor but shows no agonistic effect, or it does so only at very high (in the millimolar range) concentrations (Blank et al., 1987; Santostefano et al., 1993 and references therein). Exposure of target cells to both dioxin and ANF or exposure of the dioxin receptor to both ligands in vitro has indicated that ANF antagonizes the effect of dioxin (Blank et al., 1987; Gasiewicz and Rucci, 1991; Berghard et al., 1992; Merchant et al., 1992; Santostefano et al., 1993). The mechanism of action of ANF remains, however, unclear. In the present study we have, therefore, investigated regulation of a minimal XRE-driven reporter gene construct by ANF in vivo and examined the effect of ANF on the DNA binding function of the dioxin receptor in an in vitro reconstituted system. Finally, dioxin receptor fragments fused to a heterologous DNA binding motif were used in transfection experiments with wild-type and mutant Arnt-deficient hepatoma cells to confer aryl hydrocarbon receptor nuclear translocator, ANF, 7,8-benzoflavone (α-naphthoflavone); BNF, 5,6-benzoflavone (β-naphthoflavone); hGH, human growth hormone; CHO, Chinese hamster ovary; DBD, DNA binding domain(s); MMTV, mouse mammary tumor virus.
investigate whether ANF-mediated responses functionally required the dioxin receptor partner factor Arnt.

MATERIALS AND METHODS

Plasmids—The reporter gene constructs pMMTV-AF (generously provided by Dr. Stefan Nilsson, KaroBio Inc., Stockholm, Sweden) and pXRE-MMTV-hGH have been described previously (Alksnis et al., 1991; Whitelaw et al., 1993b). Construction of pGemArnt (Whitelaw et al., 1993a), pArntΔHLH (designated ArntΔHLH-2 in Mason et al. (1994)), and pMT-DsRed/BS has been described previously. The metallothionein promoter-driven vectors pMT-DBD, pMT-DBD/83-805, and pMT-DBD/83-839 (Whitelaw et al., 1993a) contain chimeric glucocorticoid-dioxin receptor derivatives spanning amino acids 1–500 (here designated DBD) of the human glucocorticoid receptor (Hollenberg et al., 1985) fused to amino acids 83–805 and 83–839, respectively, of the murine dioxin receptor (Burbach et al., 1992; Ems et al., 1992).

Cell Culture and Transfections—Hepa 1c1c7 cells and a phenotypically dioxin receptor nuclear translocation-deficient variant of it (Hepa c4) were kept in an atmosphere of 6% CO2 in minimal essential medium supplemented with 10% fetal calf serum, 2 mm l-glutamine, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml. CHO cells were cultured in Ham’s F-12 medium with the same supplements as for Hepa 1c1c7 cells. All media and supplements were from Life Technologies, Inc. For transient transfections, cells were seeded at a density of 10^5/35-mm dish and grown to ~50% confluence (approximately 20 h). Reporter gene constructs (1 μg) were transfected with 5 μg of DOTAP (Boehringer Mannheim) or 10 μl of lipofectin (Life Technologies, Inc.) in the absence or presence of metallothionein promoter-driven expression vectors (1 μg) in 1 ml of medium as described by the manufacturer. After 12 h of transfection, the medium was replaced with fresh medium containing the indicated concentrations of dioxin receptor ligands dissolved in dimethyl sulfoxide (final concentration of dimethyl sulfoxide, 0.1% [v/v]).

Ligand treatment was continued for 72 h. Secreted alkaline phosphatase activity was determined by a colorometric assay as described previously (Göttlicher et al., 1992). Secreted hGH levels were assayed by a radioimmunoassay (Pharmacia Biotech Inc.).

Electrophoretic Mobility Shift Assay—Gel mobility shift analysis of the DNA binding activity of the Hepa 1c1c7 dioxin receptor was performed as described previously (Happgold et al., 1989). To this end, a cytosolic extract was prepared from untreated Hepa 1c1c7 cells in extraction buffer (20 mM Tris, pH 7.4, 1% [w/v] glycerol), 1 mM EDTA, 2 mM 2-mercaptoethanol) as described (Wilhelmsson et al., 1990). Treatment with TCDD (Chemsys Laboratories, Lenexa, KS) or ANF (Serva, Heidelberg, Germany) dissolved in dimethyl sulfoxide was for 2.5 h at 25°C. In experiments where antisem were included in the reaction mixture, it was added together with the Hepa 1c1c7 cytosolic extract.

Dioxin Receptor and Arnt Antisera—Peptides corresponding to amino acids 12–13 (Burbach et al., 1992; Ems et al., 1992) of the murine dioxin receptor or amino acids 39–58 (Hoffman et al., 1991) of human Arnt were used to generate specific antisera in rabbits. Briefly, the peptides were coupled to ovalbumin via a cysteine residue added to their C termini using a heterobifunctional cross-linking reagent (SPDP, Pharmacia). Conjugates were injected into rabbits, and serum was collected 10–14 days after dosing. A peptide corresponding to amino acids 762–777 of the human glucocorticoid receptor (Hollenberg et al., 1985) was used as control in epitope competition experiments.

In Vitro Expression of Dioxin Receptor, Wild-type, or Mutant Arnt Proteins—Dioxin Receptor, wild-type, or mutant Arnt mRNA was synthesized in vitro from pDR/ATG/BS, pGemArnt, or pArntΔHLH using T3 or T7 polymerase and subsequently translated in rabbit reticulocyte lysates (Promega, Madison, WI) in the presence of either [35S]methionine or 20 μM unlabeled methionine under conditions suggested by the manufacturer.

Safety Precautions—Due to the toxicity of dioxin receptor ligands, their use requires special handling as outlined previously (Wilhelmsson et al. 1990 and references therein). Contaminated materials were disposed of by high temperature incineration.

RESULTS AND DISCUSSION

Agonistic and Antagonistic Effects of ANF on Activity of a Minimal XRE-Driven Reporter Gene Construct—To investigate effects of ANF on dioxin receptor function we transiently transfected wild-type Hepa 1c1c7 cells with the XRE-containing pXRE-MMTV-hGH reporter gene. As schematically represented in Fig. 1A, this reporter gene contains a minimal mouse mammary tumor virus (MMTV) promoter in which endogenous glucocorticoid response elements have been substituted with a single XRE element of the cytochrome P4501A1 promoter. B, reporter gene pXRE-MMTV-hGH was transfected into wild-type Hepa 1c1c7 cells. Cells were treated as indicated for 72 h. Levels of secreted hGH were measured by radioimmunoassay. hGH secretion in vehicle-treated cells was arbitrarily set to 1. Results from a representative experiment are shown.

FIG. 1. Transient transfection of an XRE-driven reporter gene into wild-type Hepa 1c1c7 cells. A, schematic representation of reporter gene pXRE-MMTV-hGH in which endogenous glucocorticoid responsive sequences have been substituted with a single XRE element of the cytochrome P4501A1 promoter. B, reporter gene pXRE-MMTV-hGH was transfected into wild-type Hepa 1c1c7 cells. Cells were treated as indicated for 72 h. Levels of secreted hGH were measured by radioimmunoassay. hGH secretion in vehicle-treated cells was arbitrarily set to 1. Results from a representative experiment are shown.
Experiments in the presence or absence of antisera against the ligand-binding dioxin receptor and Arnt-treated cytosol of Hepa ccl7 cytosol. Extract from nontreated Hepa ccl7 cells was treated with dioxin or ANF as described above, and ligand-binding activity of the extract treated with vehicle dimethyl sulfoxide (DMSO) was analyzed in the presence of preimmune serum. By analogy, formation of the ANF-induced XRE complex was specifically inhibited by the receptor antibodies, and Arnt antibodies retarded the ligand-dependent XRE complex. The specificity of the antibodies was examined in competition experiments employing the synthetic dioxin receptor and Arnt peptides that were used as epitopes for immunization, and an unrelated peptide of similar length and charge spanning a fragment of the hormone binding domain of the glucocorticoid receptor. In the presence of the dioxin receptor peptide, no inhibition of DNA binding activity by the dioxin receptor antibodies was observed. However, the antibodies produced a supershifted protein-DNA complex by the Arnt antibodies. Conversely, the Arnt peptide inhibited formation of a supershifted protein-DNA complex by the Arnt antibodies. However, the antibodies produced the supershift in the presence of the dioxin receptor and glucocorticoid receptor peptides (Fig. 2B, compare lanes 9–13).

We next reconstituted activation of the dioxin receptor by ANF using in vitro expressed dioxin receptor and Arnt proteins. In contrast to a recent report (Dolwick et al., 1993), these proteins did not show any XRE binding activity when incubated together in the absence of ligand (Fig. 3B, lane 5). However, ANF treatment induced XRE binding activity of the dioxin receptor-Arnt complex (lane 6). In control experiments, a narrow Arnt deletion mutant, ArntΔbHLH, which lacks the minimal receptor function by a-Naphthoflavone.
Regulation of Dioxin Receptor Function by α-Naphthoflavone

FIG. 4. Regulation of chimeric glucocorticoid-dioxin receptor constructs by TCDD and ANF. A, a schematic representation of chimeric receptor constructs and the reporter gene construct pMMTV-AP (top). Chimeras contain the N-terminal 500 amino acids of the human glucocorticoid receptor (hGR), including the transcriptional activation domain (TA) and the DNA binding domain (DBD), fused to amino acids 83–605 or 83–593 of the murine dioxin receptor (mDR). Dioxin receptor structures lack the N-terminal bHLH motif of the receptor but contain a region with homology to Arnt and the Drosophila proteins Per and mal bHLH domain (as schematically represented in Fig. 3A) and fails to dimerize with the dioxin receptor (Mason et al., 1994), did not enable the dioxin receptor to bind to DNA in the presence of ANF (Fig. 3B, compare lanes 7 and 8). We conclude from these experiments that the ANF-occupied dioxin receptor is competent to recruit the Arnt partner factor, resulting in XRE binding activity, and that both ANF and dioxin treatment of the receptor resulted in stimulation of receptor-Arnt heterodimerization. In strong support of this model, the Arnt deletion mutant ArntΔbHLH lacking the minimal bHLH dimerization motif failed to induce the DNA binding activity of the ANF-stimulated dioxin receptor in a reconstituted cell-free system (Fig. 3B). Finally, in agreement with the functional data in vivo (Fig. 1), the employed dose (1 μM) of ANF was less potent than 10 nM dioxin in inducing XRE binding activity in vitro by the receptor-Arnt heterodimer (Fig. 2A), possibly due to a lower efficacy in recruitment of the Arnt partner factor.

Interestingly, we failed to detect any differences in the relative mobility of the XRE complex generated by either the ANF- or the dioxin-activated dioxin receptor-Arnt heterodimers, arguing that no conformational difference in the activated receptor forms was detectable by this method. In contrast, binding of certain antiestrogens and antiprogestins to the estrogen and progesterone receptors, respectively, has been reported to result in receptor-target DNA complexes with different relative mobilities as compared with complexes generated by agonist-activated receptors (Kumar and Chambron, 1998; Brown and Sharp, 1990; Meyer et al., 1990; Klein-Hitpass et al., 1991; Beck et al., 1993). Antagonism of Dioxin Receptor Function by ANF Does Not Require bHLH Dioxin Receptor Partner Factor Arnt—Our results indicate that the ANF-occupied dioxin receptor is transcriptionally active in vivo (Fig. 1B) and is able to recruit the Arnt co-factor via the bHLH motif to acquire XRE binding activity in vitro (Figs. 2 and 3). To address the mechanistically important question of whether dioxin receptor-Arnt dimerization is a target for the antagonistic activity of ANF, we used chimeric glucocorticoid-dioxin receptor derivatives containing the first 500 amino acids of the human glucocorticoid receptor fused to various fragments of the murine dioxin receptor. As schematically shown in Fig. 4A, the employed glucocorticoid receptor fragment spans the major transactivation (TA) (Giguère et al., 1986; Hollenberg and Evans, 1988) and DNA binding domains (DBD) but not the C-terminal hormone binding domain of the receptor. This glucocorticoid receptor derivative (here termed rDBD) shows constitutive transcriptional activity (Godowski et al., 1987). Importantly, the dioxin receptor fragments that were fused to rDBD all lack the very N-terminal bHLH motif of the dioxin receptor.

Upon transient transfection into CHO cells, the chimeric glucocorticoid-dioxin receptor derivatives pMT-rDBD/83–805 and pMT-rDBD/83–593 show repression of the activity of the glucocorticoid response element-driven reporter gene pMMTV-AP. However, this negative regulation is reversed following

Sim (PAS). Q denotes a glutamine-rich region with similarity to transcriptional activation domains of other transcription factors. B, Cotransfection of pMT-rDBD/83–805 and pMMTV-AP into CHO cells. Cells were treated with the indicated concentrations of ligand for 72 h prior to analysis. In control experiments, CHO cells were cotransfected with expression vector lacking insert (pCMV) and the reporter gene. Results from a representative experiment are shown. C, Cotransfection of pMT-rDBD/83–593 and pMMTV-AP into CHO cells. Cells were treated as above. All columns represent cotransfection of pMMTV-AP with pMT-rDBD, pMT-rDBD/83–593, or pMT-rDBD/83–805 as indicated. Results from a representative experiment are shown. In panels B and C, phosphatase activity in the cell medium of vehicle-treated cells was arbitrarily set to 1.
treatment with dioxin (Whitelaw et al., 1993a) (Fig. 4, B and C). This effect was observed with both chimeric receptor forms. The dioxin receptor residues deleted in the shorter construct (amino acids 593–805 of the murine dioxin receptor) contain a glutamine-rich region bearing similarity to transcriptional activation domains in other transcription factors (Ema et al., 1992; Burbach et al., 1992; Mitchell and Tjian, 1989). The ligand binding domain of the dioxin receptor (contained within amino acids 230–421) (Dolwick et al., 1993; Whitelaw et al., 1993a) is, however, intact, demonstrating that this region of the dioxin receptor confers dioxin regulation upon a heterologous transcription factor (Whitelaw et al., 1993a). Treatment with 1 μM ANF resulted in no or a very minor induction of chimeric receptor activity (Fig. 4, B and C). When both ANF and dioxin were administered together, ANF suppressed dioxin induction in a dose-dependent manner (Fig. 4, B and C). A dioxin receptor agonist, BNF (which is structurally very similar to ANF), induced expression of the reporter gene irrespective of the chimeric receptor construct used. Unlike ANF, BNF did not antagonize the effect of TCDD in co-administration experiments (data not shown). In conclusion, the antagonistic effect of ANF is observed in dioxin receptor derivatives lacking the bHLH motif, and the 212 most C-terminal amino acids of the dioxin receptor (possibly harboring a transactivation domain) (Whitelaw et al., 1993a) are not necessary for the antagonistic activity.

Given the background that deletion of the bHLH motif of Arnt abrogates dimerization with the dioxin receptor in co-immunoprecipitation assays (Whitelaw et al., 1993b; Mason et al., 1994) and detectable interaction in DNA binding assays (Fig. 3B), there is reason to believe that the bHLH dioxin receptor deletion mutants do not functionally interact with Arnt. However, it has recently been shown that the PAS domain of the Dro sophila Per protein functions as a protein dimerization motif (Huang et al., 1993). A similar PAS domain (schematically represented in Fig. 4A) is also present in the dioxin receptor and Arnt, raising the possibility that the bHLH region may be the only motif involved in dimerization between the two proteins.

To unambiguously test the role of Arnt, if any, in the antagonistic activity of ANF, we cotransfected pMT-tDBD/83–805 together with the GRE-containing reporter gene into the mutant hepatoma cells Hepa c4, which are deficient in Arnt function (Hoffman et al., 1991). These cells do not mediate any dioxin induction of XRE-driven reporter genes including pXRE-MMTV-lGH (Whitelaw et al., 1993b and references therein). In contrast, the MMTV promoter reporter gene enhanced in a dose-dependent manner the reporter gene activity was increased by approximately 6-fold upon treatment with dioxin (1 nM). Treatment with 1 or 10 μM ANF did not significantly alter the activity of the reporter gene. When TCDD (1 nM), and ANF (1 or 10 μM) were added together, ANF completely inhibited the induction response observed with dioxin treatment alone (Fig. 5). Since agonistic effects of ANF become apparent when very high doses are used (Santostefano et al., 1993; Berghard et al., 1992), we also tested the effect of 50 μM ANF on the functional activity of the chimeric receptor construct. In our system, this concentration had no effect on expression of the reporter gene. Furthermore, as expected, BNF had an agonistic effect when added alone and did not significantly decrease the response to dioxin of the chimeric receptor when the two compounds were added together (data not shown). Taken together, the present data strongly argue that Arnt is not required for the antagonistic activity of ANF and that dioxin receptor-Arnt dimerization is not a primary target for the antagonistic action of ANF. Dioxin receptor motifs located between amino acids 83 and 805 and, thus, including the PAS domain, appear rather to contain the structural determinants for this effect to occur. We have observed that this region mediates both dioxin binding and stable association with hsp90 (Whitelaw et al., 1993a). It is, therefore, possible that ANF is less efficient than TCDD in promoting release of hsp90 from the ligand binding dioxin receptor subunit. Alternatively, we cannot rule out the possibility that the dioxin receptor contains at least one ligand-dependent transcriptional activation domain between amino acids 83 and 805 and that ANF is incapable of inducing its activity. In a similar fashion, it has been shown that the hormone-dependent transcription activation function, TAF-2, of the progesterone receptor is inactive in the presence of the anti-hormone RU486 (Meyer et al., 1990). However, in analogy to our limited understanding of ANF action, the detailed mechanism of glucocorticoid and progesterone receptor antagonism by RU486 is not yet understood, and several mechanisms including effects prior to, during, and subsequent to activation of the DNA binding activity of these receptors have been proposed to mediate antagonism by RU486 (for review, see Baulieu (1991)). Clearly, the present chimeric receptor system provides a tool that will be helpful in the identification and dissection of dioxin receptor structures that mediate the antagonistic activity of ANF.

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