

scription factor with a bHLH motif similar to that seen for cMyc and MyoD [41,42]. Expression experiments demonstrated that AhR exogenously expressed from cDNA could be activated by TCDD or 3MC to form a heterodimer with Arnt, resulting in activation of XRE binding and transactivating activities. DNA footprint analysis and GMSA revealed that binding of Sp1 to the BTE facilitated the binding of the AhR/Arnt heterodimer to the XRE by physical interaction and vice versa, cooperatively enhancing expression of the *CYP1A1* gene [38]. The AhR/Arnt heterodimer bound to the XRE sequence and, in turn recruited CBP or P300, a HAT coactivator, to the C-terminal activation domain of Arnt [43]. Transient DNA transfection assays using a XRE-driven reporter gene demonstrated that the coactivators SRC-1 and RIP-140, the retinoblastoma protein Rb, PML, and Nedd8 interact with AhR to enhance reporter gene expression [9]. ChIP assay confirmed and extended the involvement of these coactivators in the inducible expression of *Cyp1A1*. These studies determined that the related HAT coactivators, SRC-1, NCoA-2, and p/CIP, all associate with the *Cyp1A1* enhancer region in Hepa-1 cells in a TCDD-dependent manner [44,45]. Injection of anti-SRC-1 or anti-p/CIP Abs into Hepa-1 cells abolished the TCDD-mediated induction of the reporter gene, clearly demonstrating that these factors function in AhR-mediated xenobiotic signal transduction as coactivators [45]. Overexpression of these cofactors revealed that SRC-1 and NCoA-2, but not p/CIP, interact with Arnt, while AhR interact with all three coactivators. In contrast to the interaction with CBP, the helix 2 domain of Arnt interacts with SRC-1 [9,45].

Typically, genomic DNA is closely packed into the unique units of chromosomal structure, called nucleosomes. These structures consist of 146 bp of DNA wound around a core of histone proteins, containing two molecules each of H2A, H2B, H3, and H4. Neighboring nucleosomes are associated through a short stretch of DNA covered by a H1 histone linker. Assembly of this fundamental chromosomal structure prevents the transcriptional machinery from gaining access to the genes involved. Experiments using micrococcal nuclease revealed that, in the absence of inducers, the 5' upstream regulatory and coding regions of *CYP1A1* exist in a silent nucleosomal configuration. A nucleosome at the proximal promoter exists in a fixed position approximately –60 to –120 bp from the *CYP1A1* transcription start site, while the other nucleosomes are positioned randomly throughout the gene. TCDD treatment disrupts nucleosomes in the promoter and the transcribed portions of the gene, relieving nucleosomal repression [46].

In association with the chromosomal remodeling that occurs during drug induction, transient DNA transfection and ChIP analysis indicated the involvement of BRG1, a component of a subset of SWI/SNF ATP-dependent chromatin-remodeling complexes, in the enhancement of *Cyp1A1* gene expression by TCDD. Exogenous expression of BRG1 potentiated AhR/Arnt-mediated reporter gene expression in a TCDD-dependent fashion in Hepa-1 cells.

Upon co-transfection with SRC-1, BRG1 restored the inducible expression of the endogenous *Cyp1A1* gene in the BRG-1- and hBrm-deficient SW13 and C33 cell lines in response to the inducer. An ATPase-deficient mutant of BRG-1, however, was unable to do so. ChIP analysis demonstrated that BRG-1 associates with the enhancer region of the *Cyp1A1* gene in vivo in a TCDD- and Arnt-dependent manner, suggesting the specific recruitment of BRG-1 by the AhR/Arnt heterodimer. These results indicate the importance of ATP-dependent chromatin remodeling in the inducible gene expression mediated by the AhR/Arnt heterodimer [47].

The recruitment of the Mediator/TRAP/DRIP/ARC multisubunit complex to the *Cyp1A1* promoter is also important for the transduction of xenobiotic signals that activate target gene expression, as revealed by ChIP analysis, DNA transfection, and RNAi experiments. ChIP kinetic analysis of recruitment of Med220 and CDK8 (subunits of Mediator/TRAP/DRIP/ARC) demonstrated that these factors associate with the enhancer XRE sequence rapidly and persistently after TCDD treatment. These kinetics followed shortly after those seen for binding of AhR and p/CIP coactivators to the XRE sequence. In contrast, *PoIII* bound to the promoter, but not to the enhancer. Depletion of Med220 from HepG2 cells by RNAi substantially reduced Med220 protein levels and inhibited endogenous AhR-mediated transcription from the *Cyp1A1* gene. Treatment of Hepa-1 cells with TCDD induced the binding of AhR and p/CIP to the enhancer sequence within 10 min. The binding of p300 and Med220 to the enhancer and the binding of *PoIII* to the promoter were detected after 15 min. After binding reached maximal value at 15–30 min, the degree of binding of each protein remained constant until 2 h after treatment [6,48]. Although a relatively large number of coactivators and mediators have been identified to be involved in AhR-dependent induction of *CYP1A1* (Fig. 3), it seems likely that additional coactivators are also involved. As *CYP1A1* is strongly induced by PAHs and HAHs, such as TCDD, and a considerable amount of information has been accumulated in regard to the transcription and chromatin remodeling factors involved, *CYP1A1* induction continues to be a good model in which to elucidate the precise mechanisms of gene regulation, including the temporal and spatial recruitment of mediators, the interactions between transcription factors, and the modification of nucleosomal structures (Fig. 3).

Degradation of the AhR

To understand the mechanisms of gene regulation, it is important to investigate both the upregulation and termination phases of inducible transcription. Recent reports have suggested that AhR is rapidly downregulated following ligand binding by degradation [49]. Experiments using the proteasome inhibitor MG132 suggested that the downregulation of AhR is mediated by the proteasome. The concentrations of AhR proteins in the nuclear frac-

tions of cultured cells are highest after 1–2 h of TCDD treatment, and then, rapidly decline with increasing time. In the presence of MG132 or lactacystin, a 26S proteasome inhibitor, TCDD treatment elicited even higher levels of AhR and Arnt proteins within nuclei at as late as 8 h. The high levels of nuclear AhR and Arnt detected in cells treated with proteasome inhibitors included increased amount of the AhR and Arnt heterodimer that is capable of binding the XRE sequence. Cells treated with a combination of TCDD and MG132 induced reporter gene expression more rapidly at a greater magnitude than cells treated with TCDD alone. The luciferase activity increased linearly throughout the 8 h time course in cells treated with TCDD and MG132, peaking at approximately a 1300-fold induction. In cells treated with TCDD alone, inducible reporter expression peaked at 5 h, with only a 100-fold induction that gradually decreased thereafter [49,50].

A number of reports investigated the subcellular location of AhR degradation. LMB is a specific inhibitor of CRM-1-mediated nuclear export. Following LMB treatment, AhR remained predominantly in the nuclei. Treatment with this inhibitor abrogated the degradation of ligand-activated AhR in the culture cells, HepG2 or Hepa-1. GMSA demonstrated that AhR in the nuclei of LMB- and TCDD-treated cells were capable of binding to the XRE sequence. These results suggest that AhR must be exported from the nucleus to be degraded [50]. A recent study using a constitutively nuclear AhR (termed DR-NLS), which contains two NLS, revealed that AhR (DR-NLS) was degraded within nuclear compartments in a 26S proteasome-dependent manner. Although the degradation of the modified AhR (DR-NLS) may differ from that of native AhR, these results suggest that AhR can be degraded within the nucleus [51,52]. Future experiment will need to address whether the AhR and Arnt heterodimer bound to the XRE sequence is degraded by proteasome while bound to the DNA or after release from the XRE sequence. The AhR repressor AhRR, whose expression is enhanced by AhR, forms a heterodimer with Arnt to replace the AhR/Arnt complex in association with the XRE sequence [53]. AhRR may play a role in releasing the AhR/Arnt heterodimer from the XRE sequence, facilitating its degradation. As the degradation of AhR is important for the regulation of AhR activity, determination of the physiological location of AhR degradation remains important for future research.

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Induction and activation of the aryl hydrocarbon receptor by IL-4 in B cells

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Abstract

It is widely known that IL-4 and IL-13 act on various kinds of cells, including B cells, resulting in enhancement of proliferation, class switching to IgE and expression of several surface proteins. These functions are important for the recognition of the various antigens in B cells and are known to be involved in the pathogenesis of allergic diseases. However, it has not been known whether IL-4/IL-13 is involved in the metabolism of various kinds of xenobiotics including 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin (TCDD), and it remains undetermined whether TCDD, an environmental pollutant, influences IgE production in B cells, exaggerating allergic reactions. We identified IL-4- or IL-13-inducible genes in a human Burkitt lymphoma cell line, DND-39, using microarray technology, in which the *AHR* gene was included. The *AHR* gene product, the aryl hydrocarbon receptor (AhR), was induced by IL-4 in both mouse and human B cells in a STAT6-dependent manner. IL-4 alone had the ability to translocate the induced AhR to the nuclei. TCDD, a ligand for AhR, rapidly degraded the induced AhR by the proteasomal pathway, although IL-4-activated AhR sustained its expression. AhR activated by IL-4 caused expression of a xenobiotic-metabolizing gene, *CYP1A1*, and TCDD synergistically acted on the induction of this gene by IL-4. However, the induction of AhR had no effect on IgE synthesis or CD23 expression. These results indicate that the metabolism of xenobiotics would be a novel biological function of IL-4 and IL-13 in B cells, whereas TCDD is not involved in IgE synthesis in B cells.

Introduction

IL-4 and IL-13, produced by various kinds of cells including T_H2 cells, mast cells, basophils, NKT cells and eosinophils, exert similar biological activities by binding to their receptors on the surface of target cells (1). IL-4 and IL-13 act on various kinds of cells including B cells, in which IL-4/IL-13 causes enhancement of proliferation together with the stimuli via the surface IgM receptor and/or CD40, class switching to IgE and IgG4 (IgG1 in case of mice) and expression of surface

proteins such as CD23, MHC class II antigen, surface IgM, CD71 and CD72 (2–5). It is thought that these events are important for the recognition of the various antigens, particularly those derived from parasites, and for the pathogenesis of allergic diseases. However, it has not been known whether IL-4 or IL-13 is involved in the metabolism of various kinds of xenobiotics, including polycyclic aromatic hydrocarbons such as 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin (TCDD), 3-methylcholanthrene

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and benzo[*a*]pyrene. Additionally, it remains undetermined whether TCDD, an environmental pollutant, influences IgE production in B cells, although a couple of reports have described that TCDD enhances IgE synthesis in B cells (6, 7).

The aryl hydrocarbon receptor (AhR) is a transcriptional factor possessing basic helix-loop-helix/Per-aryl hydrocarbon receptor nuclear translocator (Arnt)-Sim motif, which binds to various kinds of xenobiotics, including TCDD (8, 9). Upon binding to the ligand, AhR translocates to the nuclei, followed by formation of the heterodimeric complex with Arnt. The AhR-Arnt complex induces expression of a number of monooxygenase genes such as cytochrome P4501A1 (*CYP1A1*), *CYP1A2* and *CYP1B1*, by binding to the xenobiotic responsive element on the target genes, playing a key role in metabolism of xenobiotics (8, 9). In addition, it has been recently reported that AhR directly affects cell cycle regulation in response to an agonist, although it is controversial whether it can inhibit or promote proliferation (10), and that AhR directly interacts with nuclear factor κ B, down-regulating its biological activities (11).

To explore the activity of IL-4 and IL-13 on B cells, we employed a microarray analysis, and it turned out that the *AHR* gene was included in the identified genes. In this article, we characterize the induction and activation mechanism of AhR by IL-4 in B cells. These results suggest that induction and activation of AhR are novel biological activities of IL-4 and IL-13 on B cells and that consequently IL-4 and IL-13 should be involved in the metabolism of xenobiotics by induced and activated AhR in B cells. In contrast, TCDD did not affect IgE production or CD23 expression by IL-4 in B cells, denying the synergistic effect of TCDD on IgE production by IL-4.

Methods

Reagents

Cycloheximide, TCDD and MG132 were purchased from Sigma-Aldrich (St Louis, MO, USA), Wako (Osaka, Japan) and Calbiochem (San Diego, CA, USA), respectively.

Cells

DND-39 cells and HepG2 cells were maintained in RPMI 1640 medium or Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 μ g ml⁻¹ streptomycin and 100 U ml⁻¹ penicillin. IL-13R α 1-transfected DND-39 cells were prepared and maintained as described before (12). PBMCs were separated from healthy human volunteers using LymphoprepTM (Axis-Shield, Oslo, Norway), and then B cells were isolated using DynabeadsTM M-450 CD19 (Dyna, Oslo, Norway), and cultured in the same way as DND-39 cells. DND-39 cells, HepG2 cells and human B cells were stimulated with 10 ng ml⁻¹ of IL-4 (Peprotech, Rocky Hill, NJ, USA) and/or 10 μ g ml⁻¹ of anti-IgM antibody (Cappel, Aurora, OH, USA) and/or 0.5 μ g ml⁻¹ of anti-CD40 antibody (Immunotech, Marseille, France) for the indicated times. Mouse B cells were isolated from spleen cells using StemSepTM B cell enrichment (StemCell Technologies Inc., Vancouver, Canada) and maintained in RPMI 1640 medium supplemented with 10% FCS, 50 μ M 2-mercaptoethanol, 100 μ g ml⁻¹ streptomycin and 100 U ml⁻¹ penicillin. Mouse B cells were

stimulated with 10 ng ml⁻¹ of IL-4 (Chemicon International, Temecula, CA, USA) and/or 20 μ g ml⁻¹ of LPS (Sigma-Aldrich).

Microarray analysis

Procedures of probe preparation and microarray analysis were performed as described before (13). The microarray analyses of complementary RNA from IL-13R α 1-transfected DND-39 cells were performed with human high-density oligonucleotide probe arrays (HG-U95Av2 Array) representing ~10 000 full-length, non-redundant genes supplied by Affymetrix. Hybridized probe arrays were read using a Hewlett-Packard GeneArray scanner (HP2500A, Hewlett-Packard, Palo Alto, CA, USA). The data were analyzed using Gene Chip software, Suite ver.4.0 (Affymetrix). Integrity of the RNA was verified before reverse transcription (RT) by visualization of the 28S and 18S ribosomal RNA bands on an agarose gel. Hybridization quality was checked by measuring the ratio of hybridization intensities of the 3'-5' regions of control genes.

RT-PCR

Total RNA was extracted by ISOGEN (Nippongene, Tokyo, Japan). The RT reaction primed with random hexamer was performed using GeneAmp RNA PCR Kit (Applied Biosystems Japan, Tokyo, Japan). The PCR reaction was performed with cDNA as a template using the indicated primers after an initial 1-min denaturation at 96°C, followed by the indicated cycles of 96°C for 1 min, the indicated annealing temperature for 1 min and 72°C for 1 min. The cycles used were 34, 31, 21, 32, 20 and 25, for human AhR, human Arnt, human GAPDH, the hemagglutinin-tagged truncated form of STAT6 (HA-STAT6DN), human β -actin and the human germline ϵ transcript, respectively. The annealing temperatures used were 58, 58, 54, 58, 62 and 55°C for AhR, Arnt, GAPDH, HA-STAT6DN, β -actin and the germline ϵ transcript, respectively. The PCR primers used were 5'-AGTCTGTTATAACCCAGAC-CAG-3', and 5'-GCATCACAACCAATAGGTGTGA-3' for AhR; 5'-GATGCAGGAATGGACTTGGCT-3', and 5'-CTTCCCTAAG-AGTTCCTGTGGCT-3' for Arnt; 5'-GAAGGTGAAGGTGGGAGT-3' and 5'-GAAGATGGTGGGATTTC-3' for GAPDH; 5'-CTAGCATGTATCCTTATGATGTTCTGATTATGCTGGTAC-3' and 5'-CCTCAGCCCCCTTCTGCA-3' for HA-STAT6DN; 5'-TCACCCACACTGTGCCCATCTACGA-3' and 5'-CAGCG-GAACCCTCATTGCCAATGG-3' for β -actin and 5'-CTGGGAG-CTGTCCAGGAACC-3' and 5'-GCAGCAGCGGGTCAAGG-3' for the germline ϵ transcript, respectively.

Immunoprecipitation and western blotting

Procedures of immunoprecipitation and western blotting were carried out as previously described (14). The samples were applied to SDS-PAGE and then electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, UK). The antibodies used for immunoprecipitation and western blotting were anti-AhR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Arnt antibody (Santa Cruz Biotechnology), anti-actin antibody (Biomedical Technologies, Stoughton, MA, USA) and anti-histone deacetylase class 1 (HDAC1) antibody (Santa Cruz Biotechnology). The proteins were visualized by enhanced

chemiluminescence (Amersham Biosciences). Fractionation of cell lysates was performed using NE-PER (Pierce Chemical Co., Rockford, IL, USA).

Mice

C57/BL6 and Balb/c mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). STAT6-deficient mice and AhR-deficient mice were prepared as described before (15, 16). All experiments were approved by the Saga University Animal Care and Use Committee.

Generation of DND-39 cells expressing the truncated form of STAT6

The plasmid encoding STAT6DN was prepared as previously described (17). This plasmid was transfected into DND-39 cells by electroporation, and the transfectants were maintained with a medium containing 2.5 mg ml⁻¹ of Geneticin (Sigma-Aldrich).

Real-time PCR analysis

Quantitative analysis of mRNA expression was performed using the ABI PRISM™ 7000 sequence detection system (Perkin-Elmer Japan, Urayasu, Japan), as described before (13). To calculate the copy numbers for each gene, standard curves were generated using a plasmid encoding that gene whose copy numbers were known. The PCR primers used were 5'-AACCAGTGGCAGATCAACCAT-3' and 5'-CCCATGCCAAAGATAATCACC-3' for human CYP1A1; 5'-CACTCTCTTTGGTTTGGGCA-3' and 5'-CTCCCCTGGAGACACCTTAA-3' for mouse CYP1A1; 5'-TCACCCACACTGTGCCATCTACGA-3' and 5'-CAGCGGAACCGCTCATTGCCAATGG-3' for human β -actin; 5'-ACTATTGGCAACGAGCGGTTTC-3', and 5'-GGATGCCACAGGATCCATACC-3' for mouse β -actin; 5'-CAATACTCCACCTCAGTTGGC-3', and 5'-GCATCACA-CCAATAGGTGTGA-3' for human AhR and 5'-GAAGGTGA-AGGTCCGAGT-3', and 5'-GAAGATGGTGATGGGATTTC-3' for human GAPDH, respectively. The TaqMan™ probes used were 5'-CCCTGATGGTGCTATCGACAAGGTGT-3' for human CYP1A1, 5'-AAAGTGCATCGGAGAGACCATTGGC-3' for mouse CYP1A1, 5'-ATGCCCTCCCCATGCCATCCTGCGT-3' for human β -actin, 5'-CCTGAGGCTCTTTCCAGCCTTCC-TTCT-3' for mouse β -actin, 5'-AGCCACCATCCATACTTGAA-TCCGG-3' for human AhR and 5'-CAAGCTTCCCGTTCTCAGCC-3' for human GAPDH, respectively.

ELISA for IgE

Mouse spleen cells were maintained in RPMI 1640 medium supplemented with 10% FCS (HyClone, South Logan, UT, USA), 1 mM sodium pyruvate (Invitrogen Corp., Carlsbad, CA, USA), 10 mM HEPES (pH 7.4), 0.1 mM non-essential amino acids (GIBCO BRL), 50 μ M 2-ME, 100 μ g ml⁻¹ streptomycin and 100 U ml⁻¹ penicillin, and incubated with 1 ng ml⁻¹ of IL-4 and 0.5 μ g ml⁻¹ of anti-CD40 antibody (BD Biosciences, San Jose, CA, USA) in the presence of the indicated concentrations of TCDD for 5 days. ELISA for mouse IgE was performed using a mouse IgE ELISA Quantitation kit (Bethyl, Montgomery, TX, USA).

Flow cytometry for CD23

Flow cytometric analysis of CD23 on spleen cells was performed using FITC-labeled anti-B220 antibody (BD Biosciences) and PE-labeled CD23 antibody (BD Biosciences). Quantitation of the surface staining was performed using FACSCalibur, and data were analyzed using Cell Quest software (BD Biosciences).

Results

Identification of IL-4- and IL-13-inducible genes in a human Burkitt lymphoma cell line, DND-39

To identify the biological activity of IL-4 and IL-13 on B cells, we used a microarray analysis. The microarray experiments were designed based on the guidelines of Minimum Information About a Microarray Experiment. To exclude the complexity related to the heterogeneity of primary human B cells, we used a human Burkitt lymphoma cell line, DND-39. Then the samples were prepared from DND-39 cells expressing IL-13R α 1 stimulated with either IL-4 or IL-13 for either 24 or 48 h. Of the 10 000 annotated genes present on the arrays, IL-4 and IL-13 augmented expression of 22 and 16 genes at 24 h, respectively, and 14 and 45 genes at 48 h, respectively. Among these genes, 11 genes at 24 h and 13 genes at 48 h were commonly up-regulated by both IL-4 and IL-13. The findings that the *IGHE* gene coding for the Ig ϵ H chain constant region and the *FCER2* gene coding for CD23 were included in the overlapped genes proved the appropriateness of this microarray analysis; it is well known that IL-4 or IL-13 induces expression of these genes (18, 19). The *AHR* gene was consistently induced by both IL-4 and IL-13 at both 24 h (8.9-fold and 7.0-fold, respectively) and 48 h (5.1-fold and 5.0-fold, respectively), raising the possibility that the *AHR* gene is a novel IL-4- or IL-13-inducible gene in B cells (data not shown).

Induction of AhR by IL-4 in DND-39 cells and human and mouse primary B cells

To validate further the result that induction of AhR was up-regulated by IL-4 or IL-13 in the microarray analysis, we analyzed expression of AhR induced by IL-4 by RT-PCR. Upon stimulation of IL-4, AhR started to be induced and reached a peak at 3 h in DND-39 cells, and then declined (Fig. 1A). In contrast, another component of the complex, Arnt, was not affected by IL-4. To address whether the induction of AhR by IL-4 in DND-39 cells required *de novo* protein synthesis, we next analyzed the effect of cycloheximide on the induction. The induction of AhR by IL-4 was not affected up to 50 μ g ml⁻¹ of cycloheximide (Fig. 1B). These results suggested that induction of AhR by IL-4 probably did not require *de novo* protein synthesis.

To address whether IL-4 up-regulates AhR production at not only the mRNA level but also the protein level, we next analyzed AhR induction by IL-4 using western blotting. IL-4 induced protein synthesis of AhR in DND-39 cells, whereas HepG2 cells constitutively expressed AhR and IL-4 did not affect the induction (Fig. 1C). Mouse spleen B cells derived from Balb/c or C57/BL6 strains also showed that IL-4 alone could enhance

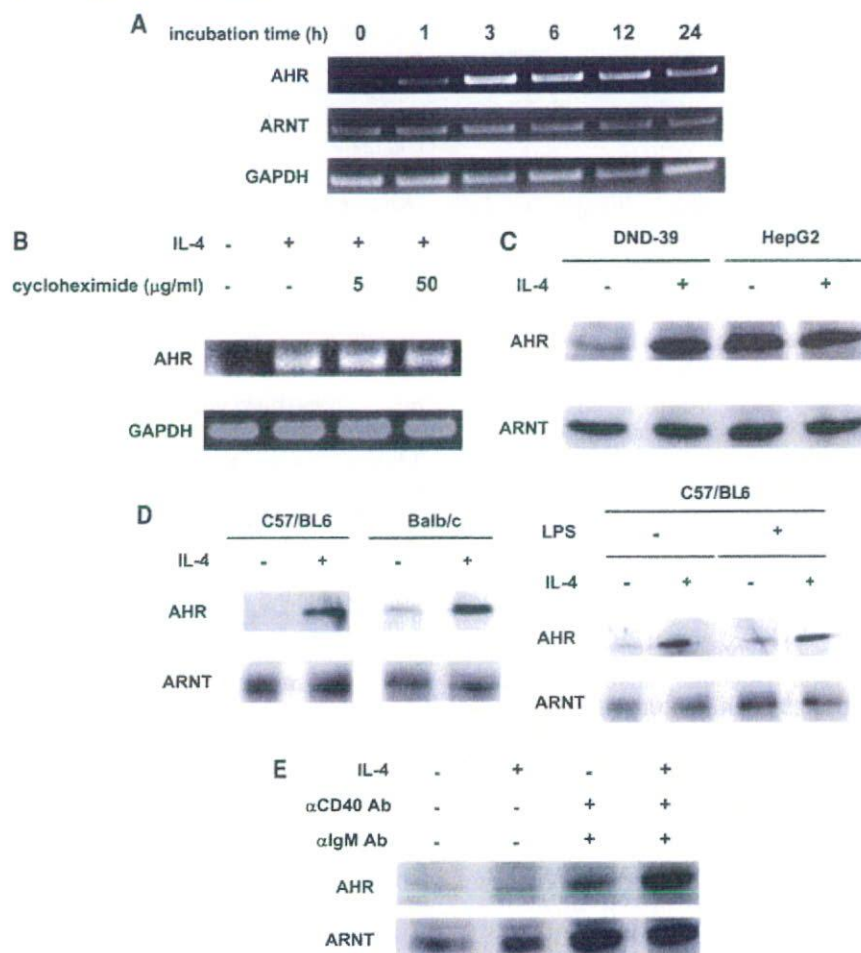


Fig. 1. Induction of AhR in B cells. (A) Total RNA was extracted from DND-39 cells stimulated with 10 ng ml^{-1} of IL-4 for the indicated times, and then RT-PCR for AhR, Arnt and GAPDH was performed. (B) Total RNA was extracted from DND-39 cells stimulated with 10 ng ml^{-1} of IL-4 for 6 h. The indicated concentrations of cycloheximide were added 30 min before the addition of IL-4. Then RT-PCR for AhR and GAPDH was performed. (C) Cell lysates were prepared from DND-39 cells and HepG2 cells stimulated with 10 ng ml^{-1} of IL-4 for 24 h, and then western blotting was performed using either anti-AhR antibody or anti-Arnt antibody. (D) Immunoprecipitates with either anti-AhR antibody or anti-Arnt antibody were prepared from spleen B cells derived from C57/BL6 or Balb/c strains stimulated with 10 ng ml^{-1} of IL-4 and/or $20 \mu\text{g ml}^{-1}$ of LPS for 24 h, and then western blotting was performed using either anti-AhR antibody or anti-Arnt antibody. (E) Cell lysates were prepared from human primary B cells stimulated with 10 ng ml^{-1} of IL-4 and/or $10 \mu\text{g ml}^{-1}$ of anti-IgM antibody and/or $0.5 \mu\text{g ml}^{-1}$ of anti-CD40 antibody for 24 h, and then western blotting was performed using either anti-AhR antibody or anti-Arnt antibody.

AhR production (Fig. 1D). LPS, which had been reported to induce AhR (20), had little effect on AhR induction. Expression of Arnt was unchanged by the stimulation. In human peripheral B cells, IL-4 alone only slightly induced AhR, whereas co-stimulants of anti-IgM antibody and anti-CD40 antibody significantly augmented the induction (Fig. 1E). Although expression of Arnt was augmented by co-stimulants of anti-IgM antibody and anti-CD40 antibody, IL-4 had no effect on the induction. We confirmed that the same amounts of proteins were loaded in each lane by Coomassie blue staining (data not shown). These results demonstrated that IL-4 had the ability to induce AhR in B cells, probably without *de novo* protein synthesis, although the co-stimuli to maximize the AhR expression were different in mouse and human B cells.

STAT6 dependency of AhR induction by IL-4

It is well known that a transcription factor, STAT6, plays critical roles for biological activities of IL-4 and IL-13 (21, 22). We next investigated whether AhR induction by IL-4 in B cells is dependent on STAT6. For this purpose, we first generated DND-39 cells expressing a truncated form of STAT6. This form lacked the SH2 domain and the activating domain for transcription; we had already shown that it acted as a dominant negative form of STAT6 (17). We used two independent clones expressing the truncated form. We confirmed that induction of the germline ϵ transcript by IL-4 was diminished in these transfectants (Fig. 2A). When these transfectants were stimulated with IL-4, AhR was not induced, whereas induction of AhR was detected in parental cells and mock-transfected

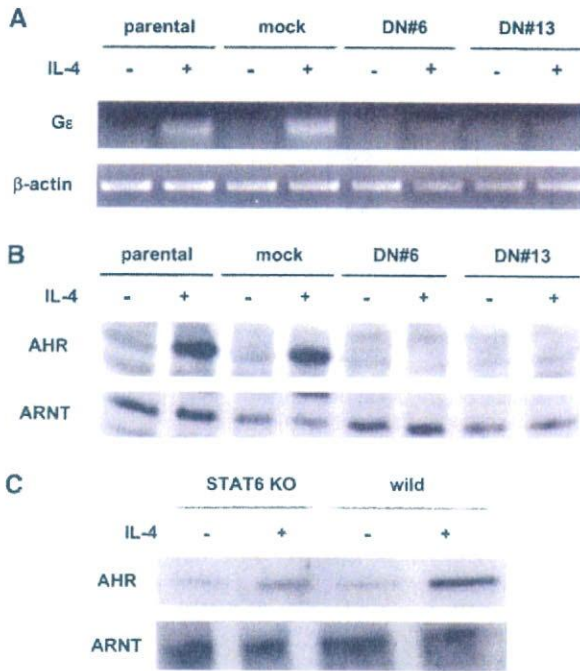


Fig. 2. Dependency of STAT6 on AhR induction in B cells. Total RNA (A) or cell lysates (B) were prepared from parental DND-39 cells, mock-transfected DND-39 cells and STAT6DN (#6 and #13) stimulated with 10 ng ml^{-1} of IL-4 for 24 h. Then RT-PCR for the germline ϵ transcript or β -actin (A) or western blotting using either anti-AhR antibody or anti-Arnt antibody (B) was performed. (C) Immunoprecipitates with either anti-AhR antibody or anti-Arnt antibody were prepared from spleen B cells derived from wild mice or STAT6-deficient mice of C57/BL6 strain stimulated with 10 ng ml^{-1} of IL-4 for 24 h, and then western blotting was performed using either anti-AhR antibody or anti-Arnt antibody.

cells (Fig. 2B). Furthermore, induction of AhR by IL-4 was significantly decreased in spleen B cells derived from STAT6-deficient mice (Fig. 2C). These results demonstrated that AhR induction by IL-4 was mostly dependent on STAT6 activation.

Effects of TCDD and IL-4 on turnover of the AhR protein in B cells

It is known that the binding of a ligand to AhR triggers degradation of AhR by the ubiquitination/proteasome pathway in hepatocytes (23–25). To explore the possibility that this would also be the case with B cells, we analyzed the effect of TCDD and also IL-4 on turnover of the AhR protein in DND-39 cells. After AhR was induced by IL-4, incubation with TCDD for 6 h caused a significant decrease of AhR (Fig. 3A). We next tested the effects of MG132, a proteasomal inhibitor, on this TCDD-induced AhR degradation. We confirmed that co-incubation of $5 \mu\text{M}$ of MG132 with 10 nM of TCDD for 12 h completely recovered AhR degradation by TCDD in HepG2 cells, as previously reported (23) (data not shown). However, the same treatment by MG132 alone caused disappearance of both AhR and Arnt in DND-39 cells, indicating the non-specific inhibitory effect of MG132 at the transcription and/or translation level (data not shown). Therefore, we incubated IL-4-stimulated DND-39 cells with $2 \mu\text{M}$ of MG132 and 10 nM of

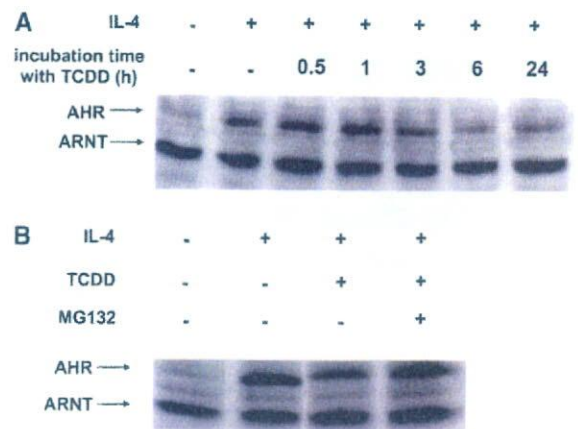


Fig. 3. Degradation of AhR proteins by TCDD. (A) Cell lysates were prepared from DND-39 cells stimulated with 10 ng ml^{-1} of IL-4 for 24 h in the presence of 10 nM of TCDD for the indicated times. TCDD was added at the indicated times before cell harvest. Then western blotting was performed using either anti-AhR antibody or anti-Arnt antibody. (B) Cell lysates were prepared from DND-39 cells stimulated with 10 ng ml^{-1} of IL-4 for 24 h. Ten nM of TCDD and $2 \mu\text{M}$ of MG132 were added 2 h before harvesting cells. Then western blotting was performed using either anti-AhR antibody or anti-Arnt antibody.

TCDD for only 2 h. The treatment of 10 nM of TCDD for 2 h degraded AhR by almost half, and co-incubation of MG132 completely recovered AhR expression (Fig. 3B). In contrast, expression of the AhR protein, which was induced by IL-4, was sustained up to 48 h (data not shown). These results suggested that TCDD rapidly degraded the induced AhR protein through the proteasomal pathway in B cells, although IL-4 alone did not cause AhR degradation.

Nuclear location of AhR by IL-4 in B cells

It has been widely believed that AhR exists in the cytoplasm at the steady state and that binding to a ligand such as TCDD leads to translocation of AhR to the nuclei, followed by formation of the ternary complex for transcription together with Arnt (8, 9). We next explored the possibility that IL-4 alone would induce translocation of AhR from the cytoplasm to the nuclei. To address this question, we fractionated the cytoplasm and the nuclei of the cell lysates and then analyzed the amounts of AhR in each fraction. We used actin and HDAC1 as the marker proteins for the cytoplasmic and nuclear fractions, respectively. Based on the amounts of these proteins, no cytoplasmic protein was detected in the nuclear fraction, whereas the cytoplasmic fraction contained some nuclear protein in our system (Fig. 4). Upon stimulation of IL-4, AhR protein was observed in not only the cytoplasmic but also the nuclear fraction in DND-39 cells, demonstrating that IL-4 was able to induce AhR expression and translocate AhR to the nuclei. Co-culture with TCDD decreased the amount of AhR in the cytoplasmic fraction in both DND-39 cells and HepG2 cells by degradation through the ubiquitination/proteasome pathway; however, the amount of AhR in the nuclear fraction was sustained or increased in both DND-39 cells and HepG2 cells. These results indicated that IL-4 not only induced AhR but also translocated it to the nuclei and that although TCDD

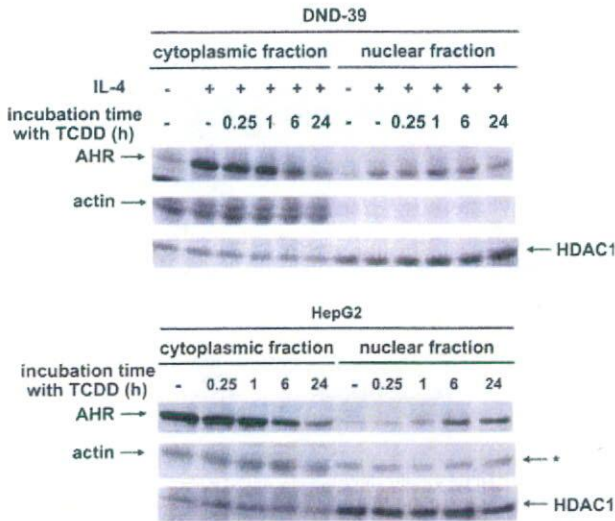


Fig. 4. Fractionation of AhR in the cytoplasm and nucleus. Cell lysates prepared from DND-39 cells and HepG2 cells stimulated with 10 ng ml^{-1} of IL-4 for 24 h in the presence of 10 nM of TCDD for the indicated times were separated into cytoplasmic and nuclear fractions. Then western blotting was performed using either anti-AhR antibody or anti-actin antibody or anti-HDAC1 antibody. The asterisk depicts a non-specific band.

degraded AhR via the proteasomal pathway, the amounts of AhR in the nuclei were not affected in the presence of TCDD in DND-39 cells.

Induction of CYP1A1 by IL-4 and/or TCDD in B cells

It has been shown that several xenobiotic-metabolizing genes such as *CYP1A1*, *CYP1A2*, *CYP1B1* and *CYP2A8* are the main targets for the transcriptional complex of AhR and Arnt (8). Based on the present findings that IL-4 induced expression of AhR and translocated AhR into nuclei, we reasoned that AhR induced and activated by IL-4 in B cells would enhance induction of these CYP enzymes. When DND-39 cells were incubated with TCDD alone, the induction of CYP1A1 was very slight (~2-fold, Fig. 5). In the presence of both IL-4 and TCDD, expression of CYP1A1 was significantly enhanced (~18-fold). It is of note that IL-4 alone had the ability to cause CYP1A1 induction (~5-fold). As the cells were harvested after incubation with TCDD for 24 h, AhR protein was almost completely diminished by TCDD (Fig. 3A).

We next analyzed induction of CYP1A1 by IL-4 in mouse spleen B cells. When mouse spleen B cells were stimulated by TCDD alone, a slight induction of CYP1A1 occurred (~2-fold, Fig. 6A). In the presence of IL-4 and TCDD, significant induction of CYP1A1 as well as DND-39 cells was detected (~9-fold). Again, IL-4 alone induced several fold of CYP1A1 (~4-fold). Induction of CYP1A1 by IL-4 tended not to be observed, but rather decreased, in STAT6-deficient mice and AhR-deficient mice. Effects of TCDD remained in STAT6-deficient mice, but disappeared in AhR-deficient mice, as expected. CYP1B1 was induced in a way similar to CYP1A1 by IL-4 and/or TCDD, but the copy numbers of CYP1B1 were extremely low compared with those of CYP1A1 (data not

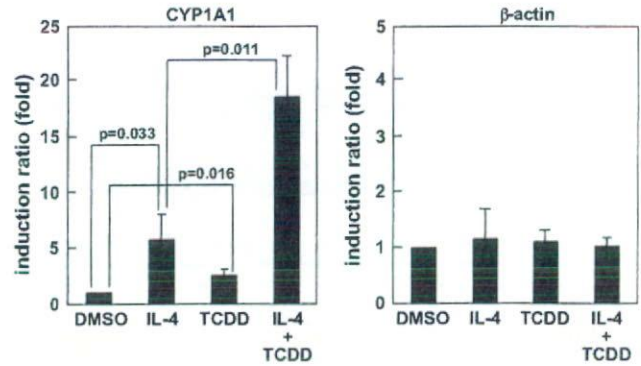


Fig. 5. Induction of CYP1A1 in DND-39 cells. Total RNA was extracted from DND-39 cells stimulated with 10 ng ml^{-1} of IL-4 and/or 10 nM of TCDD for 24 h. Then real-time PCR analysis for CYP1A1 and β -actin was performed.

shown). No induction of CYP1A2 was observed in mouse B cells (data not shown). β -Actin was slightly induced by IL-4 (~2-fold, Fig. 6B), and expression of ARNT was not affected by either IL-4 or TCDD (Fig. 6C). These results clearly demonstrated that AhR induced and translocated to nuclei by IL-4 in B cells was functional, inducing expression of a xenobiotic-metabolizing gene, *CYP1A1*, predominantly in B cells, and that TCDD exerted a synergistic effect on this induction.

Effect of AhR on IgE synthesis and CD23 expression

IgE synthesis and CD23 expression are major well-known functions of IL-4 on B cells. It has been already reported that TCDD, an environmental pollutant, enhances IgE synthesis in B cells (6, 7). To explore the possibility that AhR induced and activated by IL-4 was involved in expression of IgE and CD23, we compared the abilities to synthesize IgE and express CD23 in wild mice and AhR-deficient mice. Although when mouse B cells were stimulated with IL-4 and anti-CD40 antibody, significant IgE synthesis was observed, 5 pM of TCDD did not affect IgE synthesis and it was not impaired in AhR-deficient mice (Fig. 7A). CD23 expression was not affected by the presence of TCDD or in AhR-deficient mice (Fig. 7B). Furthermore, neither was human IgE synthesis induced by IL-4, anti-IgM antibody and anti-CD40 antibody affected by the co-culture with up to 1 nM of TCDD, nor was CD23 expression by IL-4 influenced by TCDD (data not shown). These results indicated that expression of AhR had no effect on IgE synthesis or CD23 expression induced by IL-4 in B cells.

Discussion

We report here our use of microarray analysis to identify IL-4- or IL-13-inducible genes in B cells, finding that the *AHR* gene was included in the list (data not shown). Because it is known that IL-4 and IL-13 play central roles in the pathogenesis of bronchial asthma (1, 4, 26), microarray technology has been applied to identify the downstream of IL-4 or IL-13 in several cells including bronchial epithelial cells, smooth muscle cells, lung fibroblasts and macrophages (13, 27–29) or in lung tissues derived from asthmatic monkeys induced by IL-4 (30).

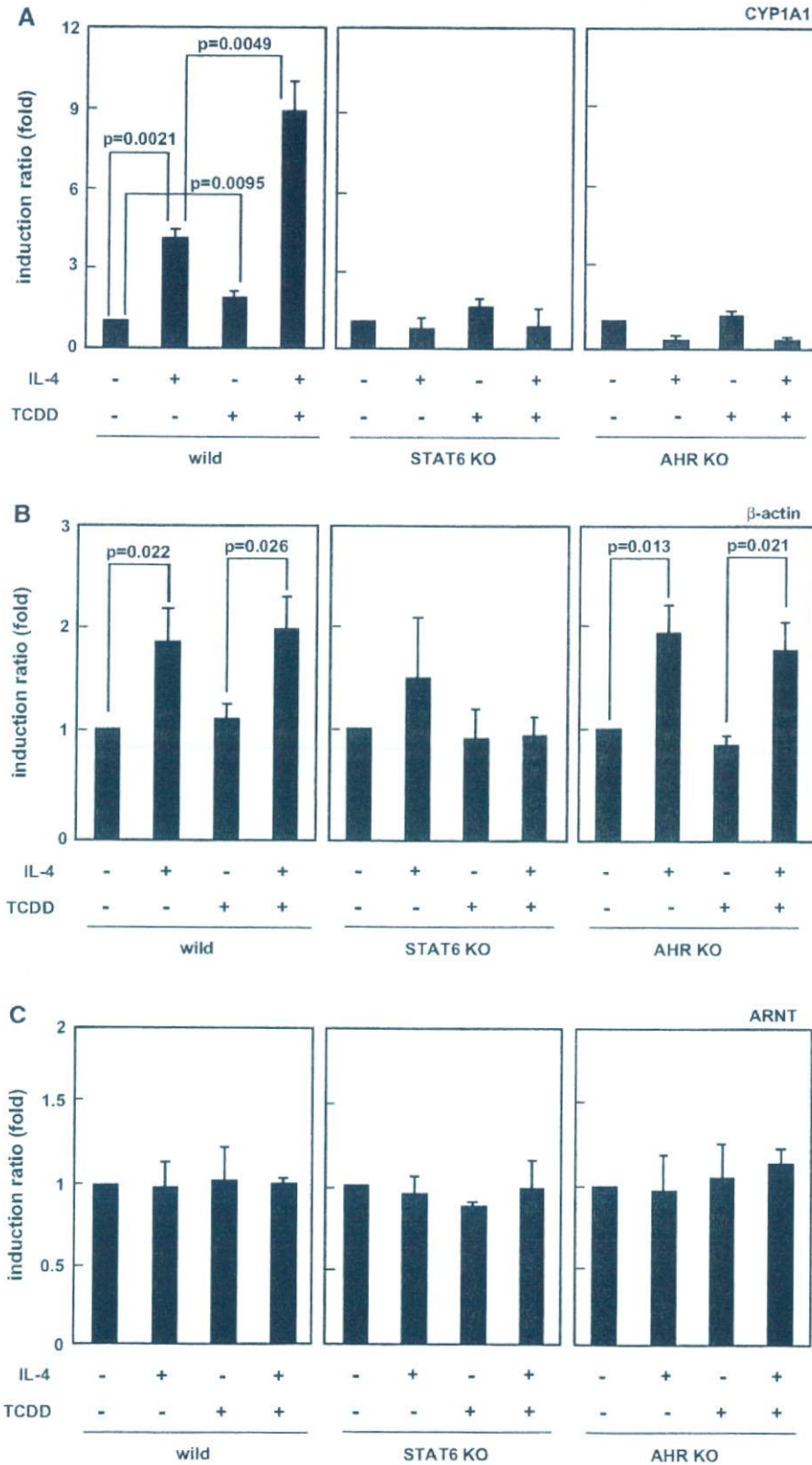


Fig. 6. Induction of CYP1A1 in mouse B cells. Total RNA was extracted from spleen B cells derived from wild mice, STAT6-deficient mice or Ahr-deficient mice of C57/BL6 strain stimulated with 10 ng ml⁻¹ of IL-4 and/or 5 pM of TCDD for 24 h. Then real-time PCR analysis for CYP1A1 (A), β-actin (B) and Arnt (C) was performed.

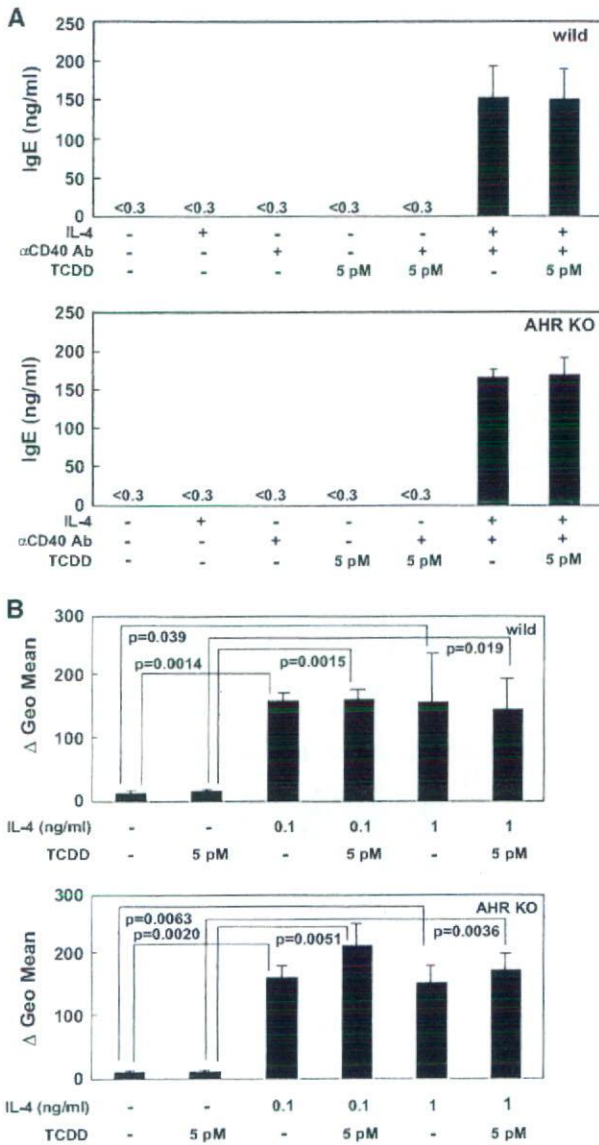


Fig. 7. Effect of AhR on IgE synthesis and CD23 expression in B cells. Spleen cells derived from wild mice or AhR-deficient mice of C57/BL6 strain were cultured with 1 ng ml⁻¹ of IL-4 and 0.5 μg ml⁻¹ of anti-CD40 antibody (only A) in the presence of 5 pM of TCDD for 5 days (A) or 24 h (B). Then the amounts of IgE in the supernatant (A) or the expression of CD23 on the surface (B) was assayed.

However, thus far, there is no application of microarray to identify IL-4- or IL-13-inducible genes in B cells. Our present microarray analysis has provided us a novel biological function of IL-4/IL-13 on B cells.

It has been already known that AhR is expressed in human tonsils (31); however, in this article, we demonstrated that AhR was induced and activated by IL-4 in B cells (Figs 1 and 4). In contrast, it has been reported that TCDD, a ligand for AhR, augments IgE synthesis in B cells (6, 7). Considering these results, it was assumed that IL-4/IL-13 and TCDD synergistically act on IgE synthesis in B cells, exaggerating allergic

reactions. However, our present results denied this possibility because TCDD had no effect on IgE synthesis and CD23 expression by IL-4 in B cells (Fig. 7). In contrast, IL-4 could induce a xenobiotic-metabolizing gene, *CYP1A1*, among a number of gene products known to be regulated by AhR (Figs 5 and 6). These data suggested that IL-4 and IL-13 would have a role in metabolizing xenobiotics, including polycyclic aromatic hydrocarbons in B cells, which is a novel biological function of these cytokines.

In this article, we demonstrated that IL-4 had an ability not only to induce AhR, but also to activate AhR (Figs 1 and 4–6). Induction of AhR was dependent on STAT6, which is known to be critical for most of the biological activities of IL-4 or IL-13 (21, 22, 32) (Fig. 2). It would be possible that STAT6 binds to the promoter region of the *AHR* gene, enhancing its induction because the 5'-flanking region of the *AHR* gene has a consensus STAT6-binding motif (TTCCTGTGAA: -1721 to -1730 nucleotides from the translation start site). The present finding that AhR induction did not require *de novo* protein synthesis (Fig. 1B) would suggest that STAT6 might bind to this site and be involved in the induction of AhR.

It has already been shown that TCDD degrades AhR via the proteasomal pathway, and ubiquitination of AhR triggers this event (23–25, 33). We demonstrated that TCDD rapidly degraded IL-4-induced AhR through the proteasomal pathway in DND-39 cells as well as HepG2 cells (Fig. 3). In contrast, IL-4 alone could cause translocation of AhR into the nuclei, sustaining expression of AhR and inducing expression of *CYP1A1* (Figs 4–6). These findings suggested that the nuclear translocation of AhR and the formation of the ternary transcriptional complex are not enough to trigger degradation of AhR but that binding of a ligand, such as TCDD, to AhR would be needed for this event. It is assumed that binding of a ligand to AhR may change its conformation, exposing the recognition site for the ubiquitination machinery. Thus, the activation mechanism of AhR is thought to be different between its ligands and IL-4 in that its ligands, but not IL-4 causes degradation of AhR, although both induce translocation of AhR into the nuclei.

In conclusion, we found that IL-4 could induce and activate AhR, inducing a xenobiotic-metabolizing gene, *CYP1A1*, in B cells. However, the induction of AhR had no effect on IgE synthesis or CD23 expression. These results indicate that the metabolism of xenobiotics by inducing and activating AhR would be a novel biological function of IL-4 and IL-13 in B cells, whereas TCDD is not involved in IgE synthesis in B cells.

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Abbreviations

AhR aryl hydrocarbon receptor
Arnt aryl hydrocarbon receptor nuclear translocator

CYP1A1	cytochrome P4501A1
HA-STAT6DN	hemagglutinin-tagged truncated form of STAT6
HDAC1	histone deacetylase class 1
RT	reverse transcription
TCDD	2,3,7,8-tetra-chlorodibenzo- <i>p</i> -dioxin

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Altered thyroxin and retinoid metabolic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in aryl hydrocarbon receptor-null mice

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Abstract To determine whether the disruption of thyroid hormone and retinoid homeostasis that occurs after exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can be mediated by the arylhydrocarbon receptor (AhR), pregnant AhR-heterozygous (AhR +/–) mice were administered a single oral dose of 10 µg kg⁻¹ TCDD at gestation day 12.5. Serum and liver were collected on postnatal day 21 from vehicle-treated control or TCDD-treated AhR +/– and AhR-null (AhR–/–) mouse pups. Whereas TCDD exposure resulted in a marked reduction of total thyroxin (TT4) and free T4 (FT4) levels in the serum of AhR +/– mice, TCDD had no effects on AhR–/– mice. Gene expression of UDP-glucuronosyltransferase (UGT)1A6, cytochrome P450 (CYP)1A1, and CYP1A2 in the liver was induced markedly by TCDD in AhR +/– but not AhR–/– mice. Induction of CYP1A1 in response to TCDD was confirmed by immunohistochemical evidence in that CYP1A1 protein was conspicuously localized in the cytoplasm of hepatocytes in the centrilobular region. Levels of retinyl palmitate were greatly reduced in the liver of TCDD-exposed AhR +/– mice, but not in vehicle-treated AhR +/– mice. No effects of TCDD on retinoid levels in the liver were found in

AhR–/– mice. We conclude that disruption of thyroid hormone and retinoid homeostasis is mediated entirely via AhR. Induction of UGT1A6 is thought to be responsible at least partly for reduced serum thyroid hormone levels in TCDD-exposed mice.

Keywords Aryl hydrocarbon receptor-null mice · Retinoid · Thyroxin · 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin · Vitamin A

Introduction

Dioxin and dioxin-like polychlorinated biphenyls (PCBs) are ubiquitously present in the environment, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent congener among a large family of dioxin compounds. Exposure to TCDD results in reproductive, immunological, and neurobehavioral toxicities, teratogenicity and carcinogenicity. Among these toxicities, effects on thyroid hormone metabolism is a major concern in humans because of epidemiological studies which suggest a possible link between these effects and environmental exposure to dioxin and related compounds (Koopman-Esseboom et al. 1994, 1996).

Thyroid hormone is required for brain development and neuronal maintenance and has metabolic functions during the fetal and early neonatal periods. Exposure to TCDD has been shown to cause morphological and functional disorders of the thyroid of laboratory animals. The most consistent effect of TCDD exposure in mammals is a marked decrease in serum T4 concentrations (Brucker-Davis 1998). Administration of a single exposure to TCDD increased remarkably the intensity of immunostaining of T4 and TSH accompanied by hyperplasia of thyroid follicular cells in Sprague-Dawley rats, suggesting that the continuous excessive secretion of TSH caused by the perturbation of the thyroid-pituitary axis by TCDD leads to proliferative changes in

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thyroid follicular cells (Nishimura et al. 2002). The thyroid hyperplasia due to disruption of thyroid hormone homeostasis was shown in the thyroid of pups born to Holtzman rat dams exposed to TCDD on gestational day (GD) 15 (Nishimura et al. 2003). However, whether the mechanism(s) responsible for functional alterations in thyroid can be mediated by the arylhydrocarbon receptor (AhR) has not been examined.

It has been established that binding to the AhR is the first necessary step for TCDD to exert its toxic effects. TCDD subsequently translocates into the nucleus, and then forms a heterodimer with the arylhydrocarbon receptor nuclear translocator. This ligand-receptor translocator complex is bound to the xenobiotic responsive element of the promoter region of target genes and accelerates transcription activity (Gonzalez and Fernandez-Salguero 1998). After production of three different colonies of AhR-null mice by homologous recombination, it is proven that AhR is responsible for most of the toxicities caused by TCDD so far examined, such as thymic involution and hepatic development (Fernandez-Salguero et al. 1996), cleft palate, and hydronephrosis (Mimura et al. 1997; Peters et al. 1999).

A very close relationship between disturbances of vitamin A homeostasis and toxicity of dioxin was first suggested because of the similar symptoms of dioxin-exposed and vitamin A-deficient animals (Innami et al. 1974). Exposure to TCDD was shown to decrease retinol and retinyl ester concentrations and the amounts of esterified storage form of vitamin A in the liver, with a significant enhancement of urinary excretion of retinoid derivatives (Brouwer et al. 1989) and in serum and kidney retinoic acid levels (Nilsson et al. 2000).

AhR seems to be involved in vitamin A homeostasis. An inverse relationship between CYP1A1 induction and hepatic vitamin A concentration suggested an AhR-mediated biochemical process in the altered vitamin A homeostasis after TCDD-dosing (Fletcher et al. 2001). Compared to AhR wild-type mice, AhR-null mice showed a three-fold increase in hepatic levels of retinyl palmitate and a three-fold accumulation in retinoic acid and retinol (Andreola et al. 1997). Differences between how various PCB congeners altered vitamin A homeostasis depended on the affinity for the AhR (Chen et al. 1992).

The activity field and signaling pathway of retinoids and thyroid hormones are closely related (Schrader and Carlberg 1994; Chin and Yen 1997), and both retinoids and thyroid hormones play critical roles in the normal development of the central nervous system (Maden et al. 1998). In women suffering from hyper- or hypothyroidism, functional disorder of the thyroid has been shown to change retinoid levels in serum (Umesh and Shobhita 1999).

Changes in vitamin A and thyroid hormone levels are well-known effect of dioxins in rodents, which have been suggested as sensitive biomarkers of exposure to dioxins. Increased oxidation and glucuronidation of retinoids by

enzymes such as CYP1A1 and UDPG (both regulated by AhR) contributes to decreased hepatic vitamin A levels following exposure to dioxins. Thyroid hormones and retinol are transported in the blood on the same carrier protein complex, transthyretin (TTR), a 55-kDa tetrameric protein having high affinity binding sites for T3 and T4 in rodents. A macromolecular complex formed by binding with retinol-binding protein (RBP) prevents renal glomerular filtration of the RBP-retinol complex. Competitive binding of hydroxylated metabolites of PCB to TTR might be responsible for a decrease in plasma levels of thyroid hormones, RBP, and retinol.

This study was designed to explore the role of the AhR in thyroid dysfunction and in the disturbance of vitamin A metabolism in response to TCDD, using AhR-null mice.

Materials and methods

Animals and TCDD treatment

AhR^{-/-} mice were generated by using a homologous recombination method as described previously (Mimura et al. 1997). Male AhR^{+/-} were backcrossed to C57BL/6 J AhR^{+/+} female mice. The genotype of each pup was determined by analyzing the presence of the mutant AhR allele by PCR with genomic DNA taken from the tail. Pups were housed in a room at 23 ± 1°C, 50 ± 10% humidity, on a 12-h light-dark cycle. The animals received laboratory rodent chow and distilled water ad libitum, and were handled with humane care under the guidelines on animal experiments of the National Institute for Environment Studies (NIES). Ten-week-old AhR^{+/-} female mice in proestrus were mated 1:1 with AhR^{+/-} males overnight, and the days when female mice had vaginal plugs the following morning were designated as day 0 of gestation. Pregnant mice (AhR^{+/-}) were administered a single oral dose of 10 µg kg⁻¹ TCDD at gestation day 12.5. Control mice (AhR^{+/-}) received an equivalent volume of corn oil. Because we did not obtain enough AhR wild-type mice (AhR^{+/+}), only AhR heterozygous (AhR^{+/-}) and AhR-null (AhR^{-/-}) mice were used in this experiment.

Serum and liver from AhR^{+/-} or AhR^{-/-} pups were collected on postnatal day 21 and analyzed. Tissues were fixed in Zamboni's solution for 24 h at 4°C and processed for immunohistological examination. For biochemical examination, tissues were snap-frozen in liquid nitrogen and stored at -80°C until further processing.

Thyroid hormone analysis

Serum total T4 (TT4) and free T4 (FT4) levels were quantified with Amerlex radioimmunoassay kits (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

RNA extraction and RT-PCR

Total hepatic RNA was extracted by using Isogen (Nippon Gene, Tokyo, Japan). The integrity of the RNA was assessed by agarose gel electrophoresis followed by ethidium bromide staining, and the quantity was determined by absorbance at 260 and 280 nm. Expression of CYP1A1, CYP1A2, UGT1A6, and β -actin was determined by reverse transcription-polymerase chain reaction (RT-PCR) using PCR primers (Table 1) for amplification according to a procedure described previously (Nishimura et al. 2002) with some modification. RT for RNA was performed in a final volume of 20 μ L containing 5.0 mmol L⁻¹ MgCl₂, 1.0 mmol L⁻¹ dNTP, 0.25 U μ L⁻¹ AMV reverse transcriptase, 0.125 μ mol L⁻¹ oligo dT-adaptor primer, 1 U μ L⁻¹ RNase inhibitor, and 1 μ g total RNA, using RNA LA PCR kit (Takara, Otsu, Japan). The RT samples were incubated at 42°C for 15 min and then at 99°C for 5 min for inactivation of RTase. PCR was subsequently performed as follows: the reaction was run at 94°C for 2 min, amplified by using temperatures of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, with 24 cycles applied for UGT1A6, CYP1A2 and β -actin and 20 cycles for CYP1A1. The PCR mixture (10 μ L) contained 2.5 mmol L⁻¹ MgCl₂, 0.25 U Takara LA Taq, 0.2 μ M of each forward and reverse primer, and 2 μ L RT products. PCR products were detected as a single band on 1.5% agarose gel in 1×TBE containing 2 μ g mL⁻¹ ethidium bromide. Band intensity was quantified by EDAS 290 system Version 3.5.3 (Kodak, Rochester, NY, USA).

Immunohistochemistry

Tissue sections of liver were stained for CYP1A1 by an indirect immunohistochemical technique described previously (Nishimura et al. 2000). Briefly, after deparaffinization and rehydration, tissue sections were heated twice for 1 min at the maximum power setting (980 W) of a microwave oven with 40–45 s boiling time (0.01 mol L⁻¹ sodium citrate buffer; pH 6.0). After microwave pretreatment, the sections were washed in PBS for 5 min and endogenous peroxidase activity was quenched by incubation in 0.3% H₂O₂ in methanol. Goat polyclonal antibody (CYP1A1, G 18: sc-9828) raised against a peptide mapping near the carboxyl terminus of CYP1A1 of mouse origin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:200 in

PBS. After incubating 2% normal goat serum with the sections, the primary antibody was incubated over the section in a humidified chamber for 1 h at 37°C. After washing sections with PBS, they were incubated for 1 h with biotinylated rabbit anti-goat IgG (BA-1000; Vector Laboratory, Burlingame, CA, USA) diluted 1:300 in PBS. The sections were then washed and incubated for 30 min with an avidin-biotinylated peroxidase complex (PK-4000, Vector Laboratory). Immunoreactions were performed using hydrogen peroxide-activated 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA). Negative controls, in which the primary antibody was replaced with normal goat IgG, did not show nonspecific staining.

Analysis of retinoids by HPLC

Hepatic retinoid analysis was performed as described by Got et al (1995) with some modification. Retinoids from liver homogenates were extracted with *n*-hexane and analyzed by HPLC (Model 2690; Waters, West Lothian, UK), monitoring fluorescence at 340 nm (excitation) and 460 nm (emission) by fluorescence detector (Model 474; Waters). Retinol, retinyl palmitate, and other retinyl esters were quantified on the basis of calibration curves using retinol or retinyl palmitate as standards.

Statistical analysis

StatView for Windows (version 5.0; SAS Institute, Cary, NC, USA) was used for statistical analysis. Values are expressed as means \pm SEM for individual groups of animals. Differences between means among experimental groups was assessed by analysis of variance; mean values for AhR +/– and AhR –/– mice were compared by Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

Results

Effects of TCDD on serum thyroid hormone concentrations and expression of TCDD-target genes in AhR +/– mice and AhR –/– mice

TCDD exposure drastically decreased serum TT4 and FT4 concentrations in AhR +/– mice compared with

Table 1 PCR primer name, sequence, and product size

Primer		Sequence (5' to 3')	Size (bp)
CYP1A1	ForwardReverse	CCATGACC GGGA ACTGTGGTCTGGTGAGCATCCTGGACA	344
UGT1A6	ForwardReverse	CTTCTGCAGGGTTTCTCTCCCAACGATGCCATGCTCCCC	875
CYP1A2	ForwardReverse	CAGTATCCAAGACATCACAAGTGTGTATCGGTAGATCTCCAG	301
β -actin	ForwardReverse	CCTCTATGCCAACACAGTAGCCACCGATCCACACAG	153

vehicle-treated control mice, but no alterations were found in TCDD-exposed AhR^{-/-} mice (Figs. 1a, b). No sexual differences in serum TT4 and FT4 levels were found as a result of different TCDD exposure.

Using semi-quantitative RT-PCR, we found that gene expression of hepatic CYP1A1 was markedly induced in TCDD-exposed AhR^{+/-} mice, but not in TCDD-exposed AhR^{-/-} mice. TCDD exposure also induced UGT1A6 and CYP1A2 expression in livers of AhR^{+/-} mice compared with vehicle-treated AhR^{+/-} mice, while no effects of TCDD were observed in AhR^{-/-} mice (Figs. 2b, c, d). Male and female animals responded to TCDD similarly for these three genes.

We next examined changes in immunohistochemical localization of CYP1A1 in the liver of TCDD-exposed AhR^{+/-} mice and AhR^{-/-} mice. No immunostaining of hepatic CYP1A1 was observed in vehicle-treated AhR^{+/-} mice (Fig. 3c) and TCDD-treated AhR^{-/-}

mice (Fig. 3d), but an increase in the intensity of cytoplasmic staining of CYP1A1 was observed clearly in TCDD-treated AhR^{+/-} mice (Figs. 3a, b). Staining for CYP1A1 was localized mainly in hepatocytes surrounding the central vein.

Effects of TCDD on hepatic vitamin A in AhR^{+/-} mice and AhR^{-/-} mice

We determined the concentrations of vitamin A derivatives that accumulated in the liver of vehicle-treated AhR^{+/-} mice, and retinoids in the liver were confirmed to consist of retinyl palmitate (approx. 90%) and retinol (Fig. 4a). TCDD treatment resulted in approximately 50% and 21% decrease in retinyl palmitate and retinol levels, respectively, in AhR^{+/-} mice in comparison with vehicle-treated AhR^{+/-} mice (Figs. 4b and c). TCDD reduced a total retinoid level in AhR^{+/-} mice to approximately 40% that of vehicle-treated AhR^{+/-} mice (Fig. 4d). Although no statistically significant changes were found between AhR^{+/-} mice and AhR^{-/-} mice, there seemed a tendency for retinyl palmitate, total retinoid, and retinol concentrations in AhR^{-/-} mice to be higher than in AhR^{+/-} mice.

Fig. 1 Effects of TCDD on serum levels of total thyroxin (TT4) (a) and free thyroxin (FT4) (b) in AhR^{+/-} and AhR^{-/-} mice. TCDD exposure induced a significant reduction of serum levels of both TT4 and FT4 in AhR^{+/-} but not AhR^{-/-} mice. Values are means \pm SEM ($n=6$). An asterisk indicates a significant difference from vehicle controls ($P<0.05$)

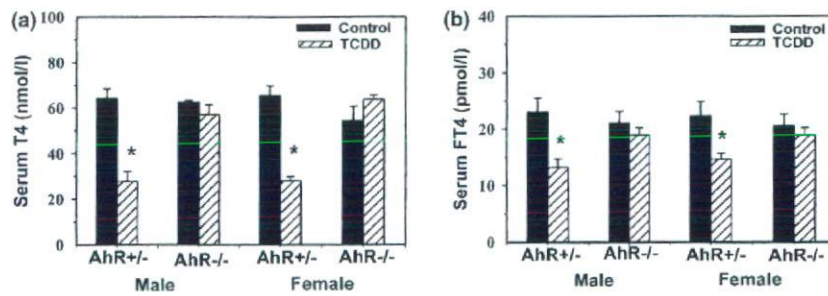
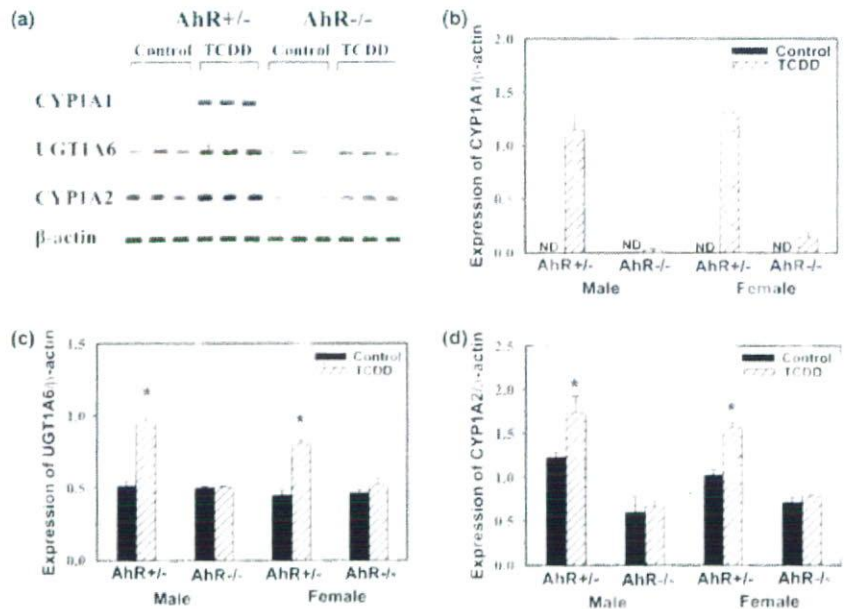


Fig. 2 RT-PCR analysis of CYP1A1 (a and b), UGT1A6 (a and c), and CYP1A2 (a and d) mRNA expression in the livers of AhR^{+/-} or AhR^{-/-} mice exposed to TCDD. Exposure to TCDD induced gene expression of CYP1A1 in AhR^{+/-} but not in AhR^{-/-} mice (both males and females). Hepatic UGT1A6 and CYP1A2A expression was induced significantly by TCDD exposure in AhR^{+/-} but not AhR^{-/-} mice. β -Actin was used to control the amount of mRNA loaded. Values are means \pm SEM ($n=4-6$). An asterisk indicates significant difference from vehicle controls ($P<0.05$)



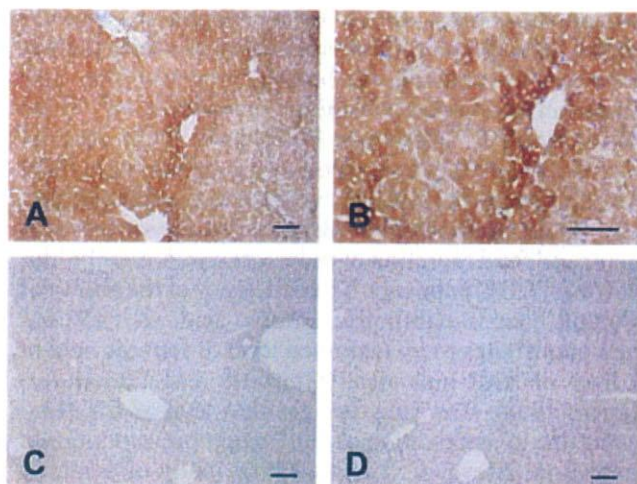


Fig. 3 Immunohistochemical location of CYP1A1 in the livers from AhR^{+/-} or AhR^{-/-} mice (postnatal day 21) exposed to TCDD. (a) Liver section from a representative TCDD-treated AhR^{+/-} mouse, and (b) higher magnification of (a). Exposure to TCDD resulted in an increase in the number of hepatocytes strongly stained with antibody specific to CYP1A1. The stained hepatocytes were located around the centrilobular region. (c) Vehicle-treated AhR^{+/-} mice showed almost no staining for CYP1A1 in the liver. (d) No staining for CYP1A1 was found in the liver from TCDD-treated AhR^{-/-} mice. Bar = 50 μ m

Discussion

It has been established that thyroid hormone plays a physiologically essential role in brain development, and disruption of thyroid hormone homeostasis has gained much attention for health risk assessment of dioxin and related compounds since epidemiological studies suggested association of cognitive ability and children's play preference with prenatal exposure to dioxin and related compounds (Vreugdenhil et al. 2002). It has been

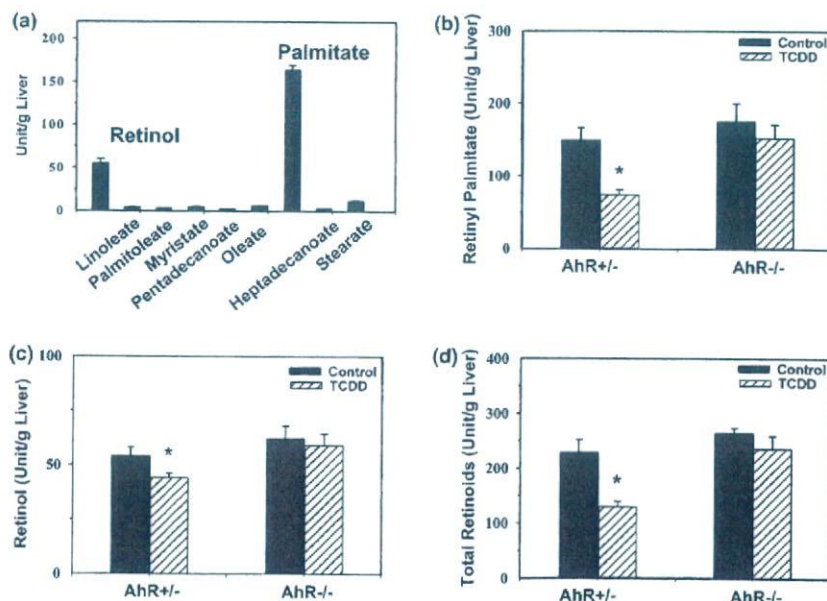
reported that thyroid hormone, T4, is conjugated with glucuronide by UGT1A1 and 1A6, a phase II drug-metabolizing enzyme, the genes of which have XRE motif(s) in the promoter region and are activated by liganded AhR-ARNT complex in the rat (Emi et al. 1996) and humans (Munzel et al. 1998). The extrathyroidal effects of TCDD on thyroid hormone turnover were studied in the rodent, and induction of UGP-glucuronosyltransferase activity by TCDD was found to be primarily responsible for reducing circulating T4 levels by accelerated biliary excretion through enhanced T4-glucuronide formation (Ritter 2000).

In this study it was clearly demonstrated by two lines of evidence that disruption of thyroid hormone homeostasis by TCDD is mediated entirely via AhR. First, serum TT4 and FT4 concentrations were not changed in TCDD-exposed AhR^{-/-} mice in contrast with a drastic reduction in AhR^{+/-} mice. Such a reduction in laboratory animals has been shown in earlier studies (Brucker-Davis 1998; Nishimura et al. 2002). Second, expression of UGT1A6 was induced in AhR^{+/-} mice but not in AhR^{-/-} mice (Fig. 2c), which resulted in reduced T4 levels in blood circulation, because of increased biliary excretion of T4 glucuronide.

The induction and consequent localization of CYP1A1 in the liver after TCDD exposure is now clearly confirmed by comparing AhR^{+/-} mice and AhR^{-/-} null mice (Figs. 2 and 3). Immunostaining for CYP1A1 was visualized in the cytoplasm around the central vein. This hepatic distribution of the protein accords well with a previous study that showed induction of CYP1A1 mRNA and its protein predominantly in centrilobular hepatocytes rather than the periportal hepatocytes, in isolated hepatocytes (Santostefano et al. 1999), and in hepatic tissue sections (Tritscher et al. 1992). Santostefano et al. (1999) reported that centrilobular hepatocytes showed a 2.7- to 4.5-fold higher

Fig. 4 Effects of TCDD on hepatic retinoid concentrations in AhR^{+/-} mice and AhR^{-/-} mice on postnatal day 21.

(a) Hepatic retinoid content of vehicle-treated control mice. (b) Hepatic retinyl palmitate content of AhR^{+/-} mice and AhR^{-/-} mice. (c) Hepatic retinol content of AhR^{+/-} and AhR^{-/-} mice. (d) Hepatic total retinoid content of AhR^{+/-} and AhR^{-/-} mice. Values are means \pm SEM ($n = 4-5$). An asterisk indicates a significant difference from vehicle-treated controls ($P < 0.05$)



concentration of TCDD compared with periportal hepatocytes. This localization of CYP1A1 probably reflects differences between TCDD distribution in liver.

Extensive studies have revealed that reduction of hepatic retinoid stores is one of the most sensitive indicators among TCDD-induced toxic manifestations. In this study we found that perturbation of vitamin A homeostasis by TCDD was clearly mediated by AhR. Pups born to TCDD-exposed AhR^{+/-} dams had a marked reduction in retinyl palmitate, retinol, and total retinoid levels compared with vehicle-treated controls whereas the levels of neither of these three substances was affected by TCDD exposure in AhR^{-/-} mice (Fig. 4). Regarding basal levels of retinoids in the liver, Andreola et al. (1997) demonstrated a 2 to 3-fold higher retinoid concentrations in AhR^{-/-} mice than AhR^{+/+} mice liver, and an intermediate value in AhR^{+/-} mice. Although no such a clear-cut difference was found in this study, at least partly because of different colonies of AhR^{-/-} mice, levels of all three species, retinyl palmitate, retinol, and retinoids, in the liver of the AhR^{-/-} pups born to vehicle-treated control dams seemed to be higher than those in AhR^{+/-} mice (Fig. 4). A plausible explanation of this difference between the two studies might be the use of different colonies of AhR^{-/-} mice.

The mechanism(s) responsible for TCDD-induced reduction of hepatic retinoid levels remain unclear. Two plausible explanations have been proposed. The first hypothesis is that TCDD inhibits enzymes, for example lecithin:retinol acyltransferase and acyl Co-A:retinol acyltransferase, responsible for converting retinol to retinyl palmitate, and increases retinyl ester hydrolase that catalyzes degradation of retinyl ester, although it is not known whether the genes of these enzymes are regulated directly in an AhR-mediated manner. Nilsson et al. (2000) demonstrated an inhibitory effect of TCDD on lecithin:retinol acyltransferase. Because, in this study, TCDD treatment was found to reduce not only retinyl palmitate, but also retinol levels in liver (Fig. 4), suppression of lecithin:retinol acyltransferase activity, if any, is unlikely to be a major contributing factor. Acyl Co-A:retinol acyltransferase is another enzyme involved in retinol esterification, but administration of TCDD did not affect hepatic Acyl Co-A:retinol acyltransferase activity except at very high dose (100 $\mu\text{g TCDD kg}^{-1}$), suggesting this enzyme is not responsible for the decrease in retinol ester level in the liver (Nilsson et al. 2000). Because Nilsson et al. (2000) also did not find an increase in hepatic retinol ester hydrolase in rats treated with 10 $\mu\text{g kg}^{-1}$, the reduction of retinol ester in the liver in this study cannot be explained by the hydrolysis pathway. These findings on TCDD exposure suggest that enzymes that catalyze either synthesis or degradation of retinyl ester do not necessarily result in the reduction of hepatic retinoid stores.

An alternative hypothesis has been sought to explain the disturbance of retinoid homeostasis by TCDD. Although several hypotheses have been proposed to explain

the impaired vitamin A kinetics by TCDD, a generally accepted view is that a decrease in retinoid stores in liver results from increased catabolism, mobilization, and elimination of hepatic retinoids (Brouwer et al. 1989; Kelly et al. 1998; Nilsson and Hakansson 2002). Retinol and retinoic acid can be metabolized via cytochrome P450 enzyme systems to inactive metabolites; subsequently, glucuronized compounds would contribute to increases in urinary and fecal excretion of polar vitamin A metabolites following TCDD exposure. The deficiency of the ability of AhR-null mice to catabolize retinoic acid via P450 enzymes contributes to an increased level of retinoic acid in the liver of AhR-null mice (Gonzalez and Fernandez-Salguero 1998). It is thus thought that lack of CYP1A1 enzyme due to inactivation of AhR might prevent vitamin A excretion. However, there is a large body of evidence that retinoids are subject to glucuronidation, leading to increased elimination into the bile (Zile et al. 1980, 1982). A decrease in hepatic stores of retinyl palmitate after TCDD exposure might result from an increased level of glucuronidation. Bank et al. (1989) found reduced retinoid levels in liver and an increase in hepatic UGT activity after TCDD exposure and verified in-vitro formation of retinyl beta-glucuronide.

Recently, Schmidt et al. (2003) observed dose-dependent elevation of all-trans-retinoic acid levels in the liver of male Sprague-Dawley rats exposed to TCDD at doses ranging from 0.1 to 100 $\mu\text{g kg}^{-1}$ body weight. In this study, 9-cis-4-oxo-13,14-dihydro-RA was drastically decreased by TCDD in a dose-dependent manner, but protein and mRNA levels of cellular retinol binding protein I (CRBP-I) in liver were not significantly altered by TCDD exposure at doses at which retinoid levels were affected, making CRBP-I an unlikely candidate to account for alterations in retinoid metabolism caused by TCDD. The authors concluded that among relevant cytochrome P450 (CYP) enzymes with potential roles in all-trans-RA synthesis and/or degradation (CYP1A1, 1A2, and 2B1/2), CYP1A1 is suggested as responsible for TCDD-induced all-trans-RA synthesis in liver and other tissues (Schmidt et al. 2003).

UGTs are enzymes that catalyze the conjugation of xenobiotics and endogenous compounds with UDP-glucuronic acid and play a crucial role in the deactivation and excretion of those compounds.

The UGT1A family is selectively induced by both MC and TCDD. The AhR is necessary for expression of MC and TCDD toxic manifestations and participates in glucuronidation of retinoids (Bank et al. 1989). Retinoid glucuronidation induced by MC was catalyzed by an MC-inducible UGT isozyme (Sass et al. 1994). Although seven isozymes are known to belong to the UGT1 family in rat (Ritter 2000), only UGT1A6 and UGT1A7 were induced by MC and PCB, which strongly supports the notion that UDP-glucuronosyltransferases are responsible for the biotransformation of retinoid in the tissue. Recent studies using AhR-null mice suggested the presence of a novel pathway of negative feedback regulation of RA synthesis from retinal via aldehyde dehydroge-

nase I (Elizondo et al. 2000). More recently, Andreola et al. (2004) reported that CYP2C39, which acts as a retinoic acid 4-hydroxylase in the liver, is down-regulated in the AhR^{-/-} mice compared with AhR^{+/+} mice, and thus responsible for disturbance of retinoid homeostasis in the AhR-null mice. However, the expression of genes that encode these enzymes were not affected by TCDD exposure in AhR^{+/+} mice. Thus, the present observation of a marked decrease in retinoid levels in the liver would have been caused by yet unidentified mechanism. Thus, it is still necessary to identify and characterize the genes and their products that are responsible for direct and indirect XRE-driven retinoid metabolism.

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