

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Induces Suppressor of Cytokine Signaling 2 in Murine B Cells

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ABSTRACT

The B cell, a major component of humoral immunity, is a sensitive target for the immunotoxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), possibly by rendering cells less responsive to antigenic or mitogenic stimulation. Potential mechanisms of TCDD action on B cells were examined in murine B cell lymphoma cells (CH12.LX) treated with 3 nM TCDD or dimethyl sulfoxide vehicle using sequence-verified cDNA microarrays. One transcript that was significantly induced by TCDD was suppressor of cytokine signaling 2 (Socs2). Changes in Socs2 mRNA levels paralleled that of Cyp1a1 with a maximal 3-fold induction observed at 4 h, as determined by quantitative real-time polymerase chain reaction. Socs2 induction seems B cell-specific, because no induction was observed in TCDD-responsive mouse hepatoma cells or human breast cancer cells. TCDD-mediated induction of Socs2 mRNA was dose-dependent and exhibited the characteristic structure-

activity relationships observed for the aryl hydrocarbon receptor (AhR) ligands 3,3',4,4',5-pentachlorobiphenyl (PCB-126), indolo[3,2-*b*]-carbazole, and β -naphthoflavone. Experiments with cycloheximide and AhR-deficient B cells indicated that Socs2 mRNA induction is a primary effect that is AhR-dependent. Western blot analysis confirmed that Socs2 and Cyp1a1 protein levels were also induced in CH12.LX cells. Promoter analysis revealed the presence of four dioxin-response elements within 1000 base pairs upstream of the Socs2 transcriptional start site, and a reporter gene regulated by the Socs2 promoter was inducible by TCDD. Promoter activity was also dependent on a functional AhR signaling pathway. These results indicate that Socs2 is a primary TCDD-inducible gene that may represent a novel mechanism by which TCDD elicits its immunosuppressive effects.

TCDD and related compounds are ubiquitous environmental contaminants that elicit a wide array of toxic and biochemical responses in a tissue-, sex-, age- and species-specific manner (Poland and Knutson, 1982). These responses are mediated by the AhR, a member of the basic helix-loop-helix/periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded family (Poland and Knutson, 1982; Denison and Heath-Pagliuso, 1998), and include a wasting syndrome, tumor promotion, teratogenesis, hepatotoxicity, modulation of endocrine systems, immunotoxicity, and enzyme induc-

tion. The proposed mechanism for mediating these responses involves ligand binding to the cytoplasmic AhR complex and the dissociation of interacting inhibitory proteins. The ligand-bound AhR then translocates to the nucleus, in which it forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator ARNT, another member of the basic helix-loop-helix/periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded family. This heterodimer then binds specific DNA elements, termed dioxin-response elements (DREs), in the regulatory regions of target genes, leading to changes in gene expression and ultimately resulting in the observed toxic and biochemical responses (Hankinson, 1995).

Evidence suggests that the adverse effects elicited by TCDD and related compounds are caused by continuous and inappropriate regulation of target genes (Denison et al., 2002). Although the mechanisms of AhR-ARNT-mediated

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; Socs2, suppressor of cytokine signaling 2; DMSO, dimethyl sulfoxide; PCB-126, 3,3',4,4',5-pentachlorobiphenyl; ICZ, indolo[3,2-*b*]-carbazole; BNF, β -naphthoflavone; IL-6, interleukin-6; PCR, polymerase chain reaction; QRT-PCR, quantitative real-time polymerase chain reaction; bp, base pair(s); DRE, dioxin-response element; Ct, threshold cycle.

changes in gene expression are fairly well established, how TCDD-elicited modulation of gene expression contributes to the observed adverse effects remains poorly understood. Well-characterized AhR-inducible genes are limited to various xenobiotic-metabolizing enzymes, including cytochromes P450 1a1, 1a2, and 1b1; however, the significance of their induction in the observed adverse responses is questionable (Schmidt and Bradfield, 1996). Recent advances in global gene expression technologies provide a comprehensive strategy to identify critical AhR-regulated target genes that contribute to adverse effects elicited by TCDD and related toxic compounds.

Immunotoxicity is one of the most sensitive responses to TCDD and related compounds. Studies with AhR-null mice and lymphoid cell lines that differ in AhR expression have confirmed that the immunotoxic effects of TCDD are mediated by the AhR (Vorderstrasse et al., 2001). In fact, TCDD is one of the most immunosuppressive agents known and elicits alterations in innate, cell-mediated, and humoral immunity (Dooley and Holsapple, 1988). A major component of humoral immunity, the B cell, has been shown to be an exceptionally sensitive immune target (Sulentic et al., 1998). It has been postulated that TCDD causes an inappropriate activation response that renders the B cells less responsive to stimulation by antigens or mitogens (Karras and Holsapple, 1994). Although many changes in the response of B cells to antigens in the presence of TCDD have been identified (Sulentic et al., 1998, 2000), the mechanisms involved remain unresolved.

To identify AhR-mediated changes in gene expression that may contribute to compromised B cell function, the effects of TCDD on gene expression in murine B cells was examined. cDNA microarray analysis identified the suppressor of cytokine signaling, *Socs2*, a member of a family of negative regulators of cytokine signaling that act via a classic negative-feedback loop (Chen et al., 2000), as a novel TCDD-inducible gene. Results from this study indicate that the induction of *Socs2* in murine B cells is a primary response to TCDD treatment. Expression of Socs proteins attenuate cytokine, growth factor, and hormone signaling (Krebs and Hilton, 2000; Greenhalgh and Hilton, 2001), and therefore AhR-mediated dysregulation of *Socs2* expression may represent a novel and potentially critical target in TCDD-elicited immunosuppression.

Materials and Methods

Chemicals. TCDD, PCB-126, and ICZ were provided by S. Safe (Texas A&M University, College Station, TX). BNF and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture. The CH12.LX B cells were originally derived from the murine CH12 B cell lymphoma, which arose in B10.H-2aH-4bp/Wts mice (B10.A \times B10.129) and the BCL-1 cell line was derived from a murine B cell lymphoma that spontaneously arose in a BALB/c mouse, and both have been characterized previously (Bishop and Haughton, 1986). Murine 225-27 B cells were obtained from Dr. Kathryn Brooks (Michigan State University, East Lansing, MI). Cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% bovine calf serum (Hyclone Laboratories, Logan, UT), 13.5 mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 50 μ M β -mercaptoethanol. Wild-type and C4 Hepa1c1c7 cells (obtained from Dr. O. Hankinson, University of California at Los Angeles, Los Angeles, CA) were

maintained in phenol red-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum (Serologicals Corporation, Norcross, GA), 100 U/ml penicillin/streptomycin (Invitrogen), 2.5 μ g/ml amphotericin B (Invitrogen), and 100 μ g/ml gentamicin reagent solution (Invitrogen). MCF-7 cells (obtained from Dr. L. Murphy, University of Manitoba, Winnipeg, MB, Canada) were maintained in low-glucose, phenol red-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 20 mM HEPES, 100 U/ml penicillin/streptomycin, 2.5 μ g/ml amphotericin B, and 2 mM L-glutamine. Cells were grown at 37°C in a 5% CO₂ humidified environment.

Cell Treatment. Cells were treated with DMSO (0.1%; vehicle control) or TCDD at the indicated concentrations and for the specified periods of time. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA was quantified (*A*₂₆₀) and assessed for purity by inspecting *A*₂₆₀/*A*₂₈₀ ratios and by visual inspection after electrophoresis of 1.0 μ g on a denaturing gel.

cDNA Microarray Analysis of Differential Gene Expression. Methods used for the construction of the microarrays, labeling of the cDNA probe, sample hybridization, and slide washing can be found online (<http://dbzach.fst.msu.edu/interfaces/microarray.html>). In brief, PCR-amplified DNA was robotically arrayed in duplicate onto epoxy-coated glass slides (Quantifoil, Jena, Germany) using an Omnigrid arrayer (GeneMachines, San Carlos, CA) equipped with 16 (4 \times 4) Chipmaker 2 pins (Telechem, Sunnyvale, CA) at the Genomics Technology Support Facility at Michigan State University (<http://www.genomics.msu.edu>). Total RNA (15 μ g) was used as a template in a reverse-transcriptase reaction in the presence of Cy3- or Cy5-dUTP to create fluorescent-labeled cDNA. Samples were purified using a QIAGEN PCR purification kit (QIAGEN, Valencia, CA). Cy3 and Cy5 samples to be cohybridized were mixed and vacuum-dried and resuspended in 32 μ l of hybridization buffer (40% formamide, 4 \times standard saline citrate, and 1% SDS) with 20 μ g of poly(dA) and 20 μ g of mouse COT-1 DNA (Invitrogen) as competitor. The probe mixture was heated at 95°C for 3 min and then was hybridized on the array under a 22 \times 40-mm coverslip (Corning Glassworks, Corning, NY) in a light-protected and humidified hybridization chamber (Corning Glassworks). The sample was hybridized for 18 to 24 h at 42°C in a water bath. Slides were then washed, dried by centrifugation, and scanned immediately at 635 nm (Cy5) and 532 nm (Cy3) on an Affymetrix 428 Array Scanner (Santa Clara, CA). Images were analyzed using GenePix 3.0 (Axon Instruments Inc., Union City, CA). Blocks were manually adjusted, and features were automatically detected and quantified as described in the manufacturer's instructions. Images were also surveyed visually to flag anomalous spots. GenePix results files, which contain raw signal-intensity values for each spot and channel, were further processed in batch using GP3, a customizable Perl script that filters spots below the limits of detection or at saturating levels, corrects for background signal, and applies a global linear normalization factor in log space to generate estimates of gene expression in each channel (Fielden et al., 2002). An independent reference design was used in which TCDD-treated samples were cohybridized with time-matched vehicle control samples. In addition, dye swaps were performed to account for potential dye-incorporation biases.

Quantitative Real-Time PCR. Total RNA (1.0 μ g) was reverse-transcribed using SuperScript II (Invitrogen) and an anchored oligo(dT) primer as described by the manufacturer. Then, 1.0 μ l of the generated cDNA was used as a template in a 30- μ l PCR reaction containing 0.1 μ M each of forward and reverse gene-specific primers, 3 mM MgCl₂, 1.0 mM dNTPs, 0.025 IU AmpliTaq Gold, and 1 \times SYBR Green PCR buffer (Applied Biosystems, Foster City, CA). All primers were designed using Primer3 (Rozen and Skaletsky, 2000). Gene names, accession numbers, forward and reverse primer sequences, and amplicon sizes are listed in Table 1. PCR amplification was conducted in MicroAmp Optical 96-well reaction plates on a PRISM 7000 Sequence Detection System (Applied Biosystems). PCR cycling conditions were as follows: initial denaturation and enzyme

activation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation protocol was also included upon completion of the amplification to ensure the absence of nonspecifically amplified products that may contribute to the overall detected signal. Each plate contained duplicate standards of purified PCR products of known template concentration that covered at least 7 orders of magnitude to interpolate relative template concentrations of the experimental samples from the standard curves of log copy number versus threshold cycle (Ct). No template controls were also included on each plate, and experimental samples with a Ct value within 2 S.D. of the mean Ct values for the no template controls were considered lower than the limits of detection. The copy number of each unknown sample for each gene was standardized to β -actin to control for differences in RNA loading, quality, and cDNA synthesis. Comparisons between vehicle and TCDD responses were analyzed using a two-way analysis of variance followed by Tukey's post test. For graphing purposes, the relative expression levels were scaled such that the expression level of the time-matched vehicle control group was equal to 1. Dose-response analysis was performed using Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

Protein Preparation and Western Blots. Western blot analysis was performed on cell lysates from CH12.LX cells. Cell lysates were prepared in 25 mM HEPES, 2 mM EDTA, and 10% glycerol with protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany), sonicated three times for 5 s, and centrifuged at 15,000g for 20 min at 4°C. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA). Cell lysate proteins were resolved by denaturing 14% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose after electrophoresis (Amersham Biosciences Inc., Piscataway, NJ). Protein blots were blocked in bovine lacto transfer optimizer buffer (4% low-fat dry milk/1% bovine serum albumin in 0.1% Tris-buffered saline/Tween 20) for 1 to 2 h at room temperature. Rabbit anti-mouse Socs2 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Donkey horseradish peroxidase-conjugated anti-rabbit IgG was purchased from Amersham Pharmacia Biotech. Immunohistochemical staining was performed as described previously (Williams et al., 1996). Detection was performed using the ECL method (Amersham Pharmacia Biotech). Protein blots were stripped and normalized by reprobing for β -actin using an anti-mouse β -actin antibody (Sigma-Aldrich). Optical density for the protein of interest was measured by densitometry using a model 700 imaging system (Bio-Rad).

Reporter Gene Construction. A 1.1-kilobase region of the mouse Socs2 promoter spanning -1103 to +10 was obtained through PCR amplification with 5'-CCCCTTCTCTTCTCCCATC-3' forward and 5'-CACAGATCGCCTGTGAAGAA-3' reverse oligonucleotides using CH12.LX genomic DNA as a template. Likewise, a 2.3-kilobase region of the mouse Cyp1a1 promoter spanning -2333 to +9 was obtained through PCR amplification with 5'-GGGGGAAGCCATGTTTATT-3' forward and 5'-CACCTTCAGGGTTAGGGTGA-3' reverse oligonucleotides. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison WI) and were then subcloned into

the pGL3-Basic vector (Promega). Promoter identities were verified by sequencing on an ABI Prism 3100 genetic analyzer (Applied Biosystems) at the Genomic Technology Support Facility on the campus of Michigan State University (<http://www.genomics.msu.edu>).

Luciferase Assays. For transient transfections, CH12.LX cells (1×10^7) were suspended in 200 μ l of culture media with 10 μ g of the Socs2 or Cyp1a1 reporter constructs and transferred to a 2-mm gap electroporation cuvette. Cells were electroporated using an electrocell manipulator (ECM 600; BTX, San Diego, CA) with the voltage set at 150 V, the capacitance at 1500 μ F, and the resistance at 72 Ω . For each construct, multiple transfections were pooled, and cells were aliquoted into a 12-well plate at 5×10^5 cells/well in 2 ml of media. Immediately after transfection, CH12.LX cells were treated with vehicle (DMSO) or TCDD for 6 h. For transient transfection experiments using Hepa1c1c7 cells, cells were seeded at 2×10^5 cells/well in 12-well tissue-culture dishes and were transfected with 0.5 μ g of the Socs2 or Cyp1a1 constructs the next day using LipofectAMINE 2000 (Invitrogen). After a 5-h incubation, Hepa1c1c7 cells were treated with vehicle (DMSO) or TCDD for 6 h. All treatments were done in triplicate. After the 6-h incubation period, cells were washed with $1 \times$ phosphate-buffered saline and lysed with $1 \times$ reporter lysis buffer (Promega). Samples were immediately frozen at -80°C. For measurement of luciferase enzyme activity, samples were thawed, and 10 μ l of sample lysate was mixed with 100 μ l of luciferase assay reagent (Promega) using an autoinjector. Luciferase activity or luminescence was measured by a Luminoskan RS luminometer (Labsystems, Vantaa, Finland) and represented as relative light units. Transfection efficiency of Hepa1c1c7 cells was normalized with respect to β -galactosidase activity, which was produced by cotransfection with 0.5 μ g of pCH110 (Amersham Biosciences). For measurement of β -galactosidase activity, 30 μ l of lysate was mixed with 100 μ l of β -galactosidase reaction buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 150 μ M β -mercaptoethanol) and 30 μ l of 4 mg/ml *o*-nitrophenyl-B-D-galactopyranoside (Sigma-Aldrich) and incubated at 37°C for 2 to 4 h. The reaction was stopped with the addition of 25 μ l of 1 M NaHCO₃, and the absorbance was measured at 420 nm in an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA).

Results

Identification of TCDD Induction of Socs2. A 3068-feature cDNA array representing 2258 unique genes was used to investigate TCDD-elicited changes in gene expression in the CH12.LX murine B cell line. This cell line has been characterized previously to express a functional AhR and has been shown to be responsive to TCDD treatment, as indicated by characteristic changes in gene expression (Sulentic et al., 1998). Several genes known to play important roles in immune function were identified as being induced or

TABLE 1
Gene names and primer sequences (5'→3') for QRT-PCR

Gene Name	Gene Symbol	Locus Link	Ref Seq Accession	Forward Primer	Reverse Primer	Product Size
Mouse						
Actin, β , cytoplasmic	Actb	11461	NM_007393	GCTACAGCTTCACCACCACA	TCTCCAGGGAGGAAGAGGAT	bp
Cytochrome P450, family 1, subfamily a, polypeptide 1	Cyp1a1	13076	NM_009992	AAGTGCAGATGCGGTCTTCT	AAAGTAGGAGGCAGGCACAA	123
Suppressor of cytokine signaling 1	Socs1	12703	NM_009896	ACTTCTGGCTGGAGACCTCA	CCCAGACACAAGCTGCTACA	100
Suppressor of cytokine signaling 2	Socs2	12701	NM_007706	TGTGCAAGGATAAACGGACA	AATGGCGAGTCGACAGAAAT	119
Suppressor of cytokine signaling 3	Socs3	12702	NM_007707	ATTCACCCAGGTGGCTACAG	CGGACCTACTGACCGAGAGA	128
Human						
Actin, β	ACTB	60	NM_001101	CATCCCCCAAGTTTCAACAAT	AGTGGGGTGGCTTTTAGGAT	125
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	1543	NM_000499	CTGCCAAGAGTGAAGGGGAAG	GGGCAGAGGAATGTGATGTT	131
Suppressor of cytokine signaling 2	SOCS2	8835	NM_003877	AAGGCTGAAGTCGCGTTTGA	AGCTTGGTTCCCTCCCACTT	132

repressed in response to TCDD, and these data are presented elsewhere (D. Boverhoff and T. Zacharewski, manuscript in preparation). One of the TCDD-inducible genes identified was Socs2, which exhibited the same induction profile as the well-characterized TCDD-responsive gene, Cyp1a1. Maximal induction for each gene was observed 4 h after TCDD treatment, and both responses were verified using quantitative real-time PCR (QRT-PCR) (Fig. 1, A and B). In general, microarrays underestimated the fold-change ratios of the mRNA transcripts compared with QRT-PCR, which revealed that Cyp1a1 and Socs2 induction was approximately 400- and 3-fold, respectively. This compression of the data has been reported previously when comparing microarray data to other, more gene-specific, measurement techniques (Yuen et

al., 2002). Data for Cyp1a1 were extremely compressed because of the inability to detect basal expression of this transcript during microarray analysis, which results in the use of background intensity levels for data interpretation. Despite the compression, the temporal expression patterns for both Cyp1a1 and Socs2 were comparable between the microarray and QRT-PCR data. The maximal induction of both Cyp1a1 and Socs2 at 4 h was followed by a decrease in transcript levels at 8 h. This response was reproducible and seems to be caused by the rapid proteolysis of the AhR in this cell line (J. Lapres, unpublished results), although effects on mRNA degradation cannot be dismissed. To investigate the effect of TCDD on other members of the Socs family, QRT-PCR analysis was used to assess transcript levels of Socs1 and Socs3.

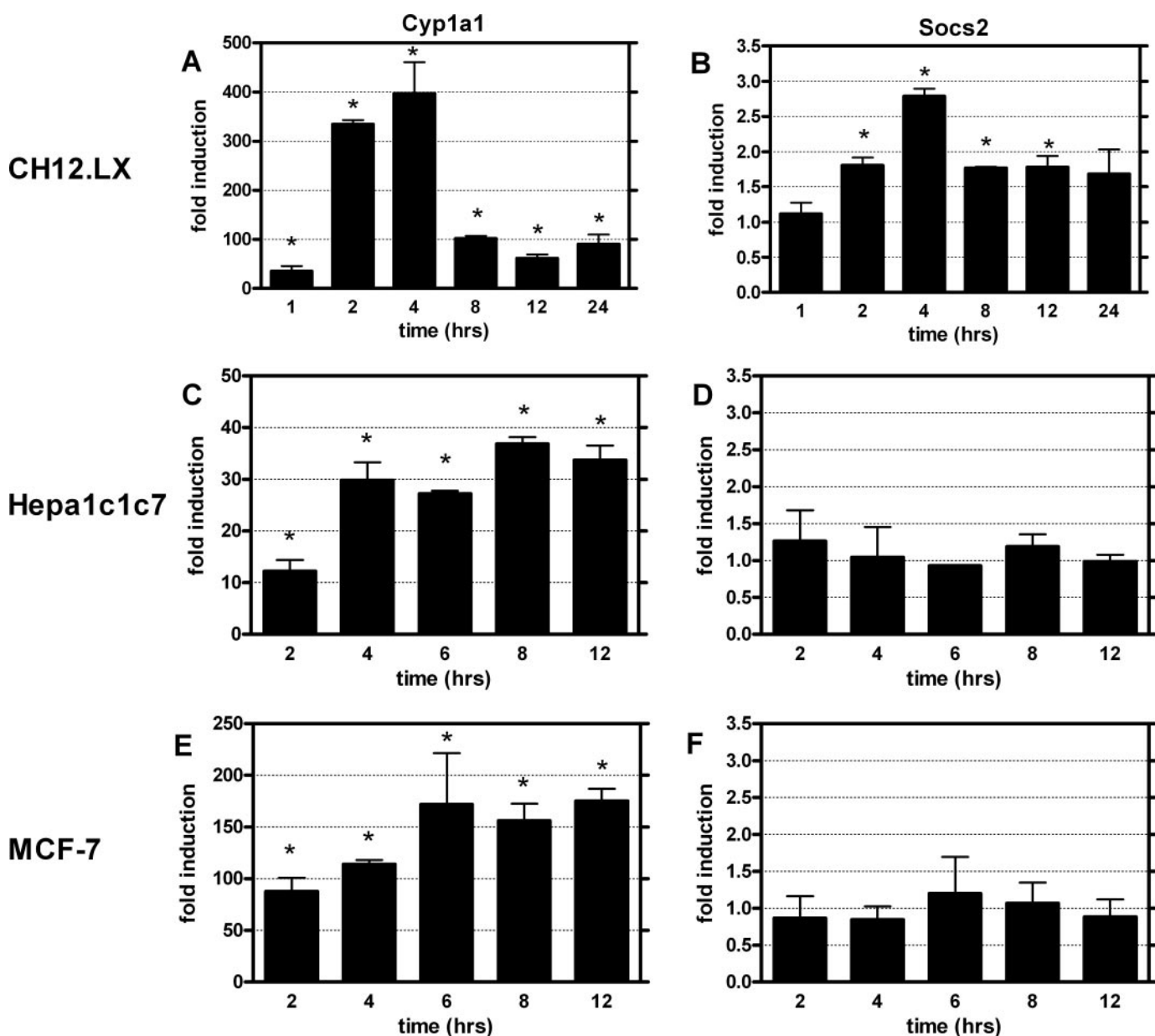


Fig. 1. TCDD induces Socs2 in CH12.LX cells but not in other AhR-responsive cell lines. Murine CH12.LX B cells, Hepa1c1c7 hepatoma cells, and human MCF-7 breast cancer cells were treated with vehicle alone (0.1% DMSO) or TCDD (3 nM) for the indicated periods of time, and transcript levels were analyzed by QRT-PCR. Fold induction values were calculated relative to time-matched vehicle controls. Cyp1a1 (A, C, and E) was induced in all three cell lines, whereas Socs2 (B, D, and F) transcripts were induced only in CH12.LX cells. Error bars represent the S.E.M. for the average fold change of at least three independent replicates, and similar results were obtained in at least three independent experiments. *, values significantly different ($p < 0.05$) from time-matched vehicle controls.

In contrast to its effects on Socs2 transcripts, TCDD had no effect on Socs1 and Socs3 transcript levels.

TCDD-induction of Socs2 mRNA was also examined in the TCDD-responsive murine Hepa1c1c7 and human MCF-7 cells. Cyp1a1 transcripts were found to be induced in both cell lines; however, Socs2 mRNA levels were not affected (Fig. 1, C–F), suggesting potential cell-type-specific regulation. TCDD treatment of murine 225-27 B cells, which also express the AhR, resulted in a Cyp1a1 and Socs2 induction profile similar to that observed in CH12.LX cells (data not shown), further implicating TCDD-mediated Socs2 induction as a B cell-specific response. It is interesting that Cyp1a1 transcripts in Hepa1c1c7 and MCF-7 cells reached maximum induction within 4 h and remained elevated, whereas Cyp1a1 mRNA levels in CH12.LX cells decreased dramatically between 4 and 8 h, indicating that AhR signaling, or Cyp1a1 mRNA, may also be subject to cell-specific regulatory influences.

AhR-Mediated Induction of Socs2 mRNA and Protein Levels. Several lines of investigation, including genetic, structure-activity relationships, and in vitro and in vivo AhR null studies, indicate that many of the effects elicited by TCDD are mediated by the AhR (Vorderstrasse et al., 2001; Bunger et al., 2003; Mimura and Fujii-Kuriyama, 2003). To confirm the role of the AhR in the induction of Socs2, the effects of structurally diverse AhR ligands were examined in CH12.LX cells. ICZ, PCB-126, and BNF all induced Cyp1a1 transcript levels in a dose-dependent manner to a maximal level of approximately 400-fold (Fig. 2A). Dose-dependent induction of Socs2 mRNA was also observed with the same rank order potency as was seen for Cyp1a1 transcripts (Fig. 2B). Ligand potency was also consistent with the relative AhR binding affinities of these ligands (TCDD > ICZ > PCB-126 > BNF) (Hestermann et al., 2000; Denison and

Nagy, 2003). Furthermore, the EC₅₀ values for each ligand were nearly identical for Cyp1a1 and Socs2 (Fig. 2C).

TCDD-mediated Socs2 induction was further investigated in the BCL-1 murine B cell line, which does not express the AhR and has been used previously to characterize AhR-dependent gene induction and TCDD-mediated suppression of lipopolysaccharide-induced IgM secretion in B cells (Sulentic et al., 1998). Cyp1a1 and Socs2 mRNA levels were induced in the CH12.LX cells, whereas no induction was observed in BCL-1 cells (Fig. 3). These results suggest that Socs2 induction is dependent on the presence of a functional AhR.

Western blot analysis of cell lysates from TCDD-treated CH12.LX cells verified the induction of Socs2 protein in response to treatment (Fig. 4). Socs2 protein was induced in a pattern similar to that observed for mRNA, with maximal induction being observed within 4 h of TCDD exposure. Cyp1a1 displayed a more sustained induction than that of Socs2, which may indicate differences in protein stability or degradation rates between these two proteins (data not shown). Together, these results confirm AhR-mediated induction of Socs2 mRNA and protein levels in response to TCDD treatment.

Induction in the Presence of Cycloheximide. The parallel induction kinetics of Socs2 and Cyp1a1 mRNA levels suggest that both responses are primary responses to TCDD that are mediated by the AhR. Cycloheximide pretreatment studies were conducted to determine whether concomitant protein synthesis was required for TCDD induction of Socs2 transcripts. Cyp1a1 mRNA levels, an established primary response, were induced more than 300-fold after treatment with TCDD alone and in excess of 500-fold when pretreated with cycloheximide (Fig. 5A). Likewise, Socs2 was induced 3.5-fold with TCDD alone and 5-fold with cycloheximide pre-

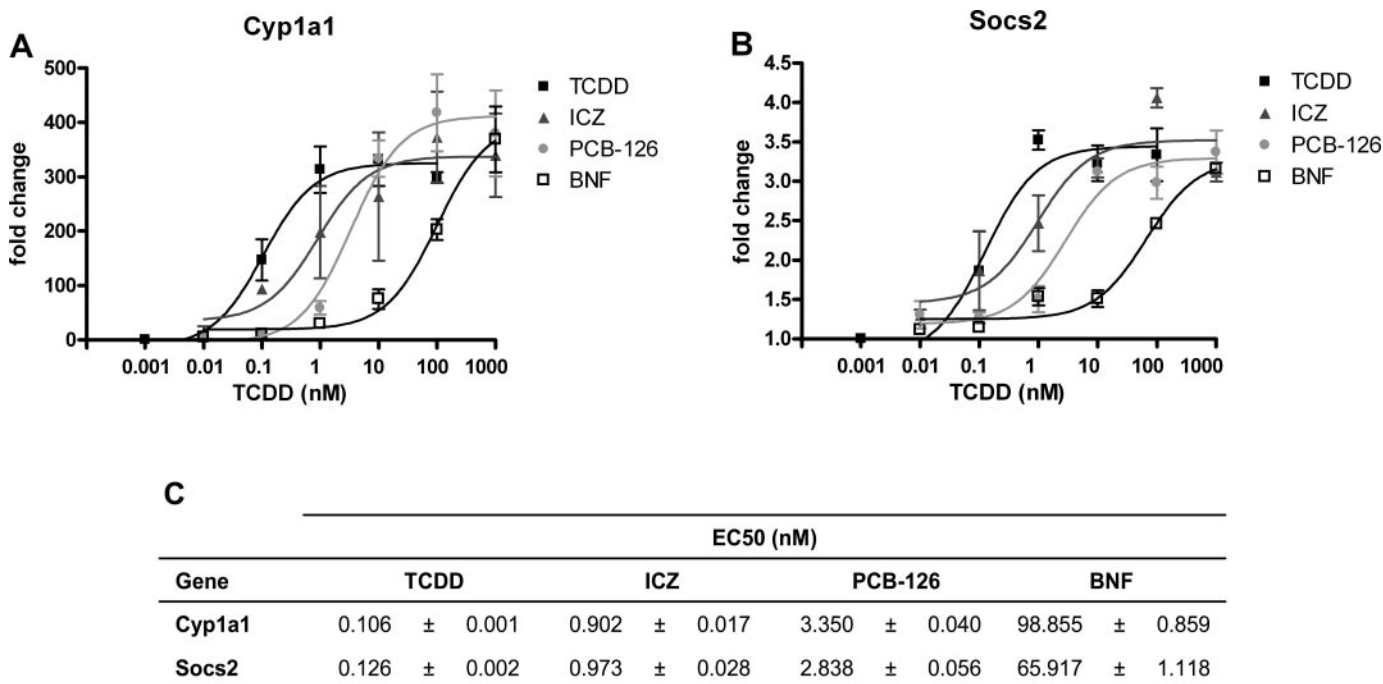


Fig. 2. Dose-dependent induction of Cyp1a1 and Socs2 transcripts by diverse AhR ligands. CH12.LX cells were treated with increasing doses of TCDD, ICZ, PCB-126, or BNF for 4 h, and transcript levels of Cyp1a1 and Socs2 were analyzed by QRT-PCR (A and B). Fold induction values were calculated relative to the vehicle control (0.1% DMSO). Dose-response curves were fitted to the data, and the EC₅₀ for each compound was determined for both Cyp1a1 and Socs2 (C). Error bars represent the S.E.M. for the average fold change of at least three independent replicates.

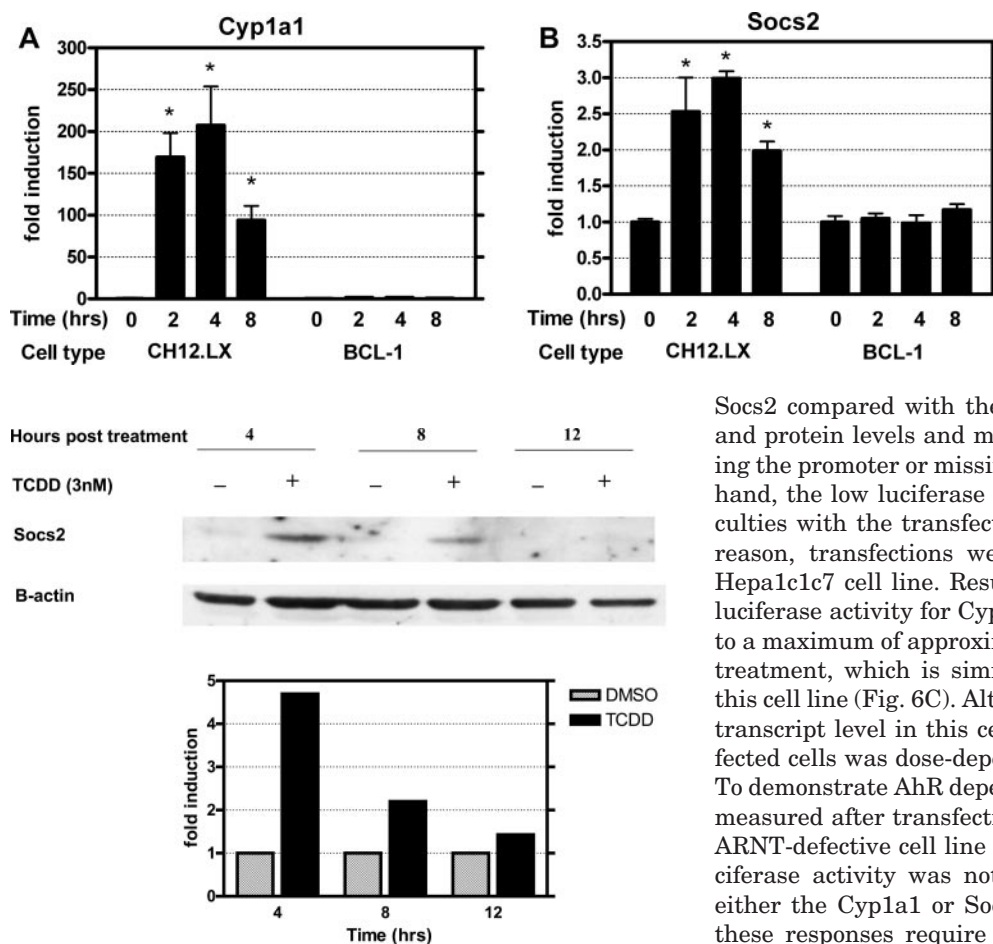


Fig. 4. Western blot analysis of Socs2 protein levels in CH12.LX cells after exposure to TCDD. CH12.LX cells were exposed to DMSO (0.1%) or TCDD (3 nM) for the indicated periods of time and were assayed for Socs2 protein levels. Socs2 protein levels were normalized to protein levels of β -actin after densitometric analysis and were expressed as fold induction relative to time-matched vehicle controls. Results are representative of three independent replicates.

treatment and TCDD (Fig. 5B). The superinduction of Cyp1a1 transcript levels in the presence of cycloheximide have been reported previously and are believed to be caused by the inhibition of ligand-induced AhR degradation (Ma et al., 2000). The parallel superinduction of Socs2 mRNA further supports the similar regulation of these transcripts.

Activity of Socs2 Promoter in Response to TCDD. TCDD-induced gene expression involves the binding of the AhR/ARNT complex to DREs in the regulatory regions of target genes. Analysis of the upstream regulatory region of the mouse Socs2 gene extracted from the University of California at Santa Cruz Genome Browser (<http://genome.ucsc.edu>, Build 2) identified four putative DREs within the first 1000 bp upstream of the transcriptional start site (−715, −613, −486, and −112 bp). To determine their activity, the −1103 to +10-bp region of the Socs2 promoter and the −2333 to +9-bp region of the Cyp1a1 promoter (positive control) were PCR-amplified and cloned into a luciferase reporter gene. Luciferase activity in CH12.LX cells transfected with the Cyp1a1 or Socs2 reporter genes was dose-dependently induced in response to TCDD treatment with maximal induction of 30- and 1.8-fold, respectively (Fig. 6, A and B). Induction of luciferase activity was lower for both Cyp1a1 and

Fig. 3. Dependence of Socs2 induction on a functional AhR in murine B cells. CH12.LX and BCL-1 murine B cells were treated with TCDD (3 nM) or vehicle (0.1% DMSO) for the indicated periods of time, and Cyp1a1 and Socs2 transcript levels were determined by QRT-PCR. Both Cyp1a1 (A) and Socs2 (B) transcript levels were induced in CH12.LX cells. Induction of these transcripts was not observed in BCL-1 cells, which do not express the AhR. Error bars represent the S.E.M. for the average fold change of at least three independent replicates. *, values significantly different ($p < 0.05$) from time-matched vehicle controls.

Socs2 compared with the induction observed for transcript and protein levels and may be caused by other factors binding the promoter or missing enhancer elements. On the other hand, the low luciferase induction might be caused by difficulties with the transfection of the CH12.LX cells. For this reason, transfections were also performed in the murine Hepa1c1c7 cell line. Results of these studies indicated that luciferase activity for Cyp1a1 was dose-dependently induced to a maximum of approximately 75-fold in response to TCDD treatment, which is similar to Cyp1a1 transcript levels in this cell line (Fig. 6C). Although Socs2 was not induced at the transcript level in this cell line, luciferase activity in transfected cells was dose-dependently induced 2.8-fold (Fig. 6D). To demonstrate AhR dependence, luciferase activity was also measured after transfection into the Hepa1c1c7 C4 cells, an ARNT-defective cell line (Hankinson, 1995). Induction of luciferase activity was not observed in C4 mutant cells for either the Cyp1a1 or Socs2 reporter genes, indicating that these responses require a functional AhR/ARNT signaling pathway (Fig. 6, E and F). The results obtained from the transfections in the CH12.LX and Hepa1c1c7 cell lines indicate that the Socs2 promoter is activated in response to TCDD.

Discussion

One of the most sensitive toxic endpoints of TCDD exposure is immune suppression. Although the toxic effects of this compound are generally accepted to be mediated by prolonged changes in gene expression via signaling through the AhR, the number of known AhR-regulated genes is small. Furthermore, even less is known about tissue-specific gene regulation by TCDD and structurally related chemicals. This study has identified and characterized Socs2 induction in murine B cells in response to TCDD treatment. Socs2 was induced both temporally and dose-dependently in a pattern similar to that of Cyp1a1. Various diverse ligands of the AhR were also able to induce Socs2 in CH12.LX cells, including ICZ, PCB-126, and BNF. It is significant that the ED₅₀ values for Socs2 induction for each of the AhR ligands were very similar to that observed for Cyp1a1, and the potency of each compound corresponded directly to the previously reported AhR binding affinity. Together, these results strongly suggested that the induction of Socs2 was mediated through the activation of the AhR signaling pathway. This notion was further supported by the lack of induction of both Cyp1a1 and Socs2 in the AhR-nonexpressing murine BCL-1 cells.

Transcript and protein levels for Socs2 were induced in a similar temporal pattern, suggesting that Socs2 activity is

largely regulated at the transcriptional level. Our findings are in agreement with a recent study which indicated that Socs proteins are short-lived, with the half-life of Socs2 determined to be 1 h (Siewert et al., 1999). The short half-life is believed to be caused by the presence of the conserved Socs box motif located on the C terminus of all Socs proteins. The Socs box motif interacts with elongins B and C, which target these proteins to the proteasome for ubiquitination and degradation (Zhang et al., 1999). The TCDD-mediated induction of Socs2 protein and its regulation at the transcriptional level

observed in the present study is consistent with known mechanisms involved in the cellular regulation of this protein.

It is interesting that TCDD induced Socs2 transcripts in CH12.LX B cells but not in Hepa1c1c7 murine hepatoma cells or MCF-7 human breast cancer cells. The reason for this profile of activity is presently unclear but suggests that AhR-mediated Socs2 regulation may be a cell- or tissue-specific response. This cell-type-specific regulation of Socs2 may conceivably be caused by cell-specific expression of various transcriptional activators and repressors that normally interact

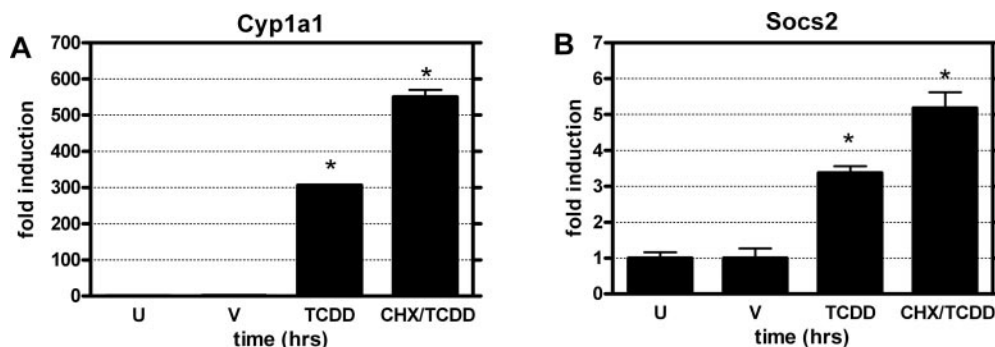


Fig. 5. Cycloheximide (CHX) does not block Socs2 induction by TCDD in CH12.LX cells. CH12.LX cells were left untreated or were treated for 4 h with TCDD or the vehicle control (0.1% DMSO). For cycloheximide studies, cells were treated with 10 μ g/ml cycloheximide for 1 h before treatment with TCDD for 4 h. Transcript levels of Cyp1a1 (A) and Socs2 (B) were determined using QRT-PCR. Fold inductions are expressed relative to vehicle controls. *, values significantly different ($p < 0.05$) from the vehicle control. U, untreated control; V, vehicle control.

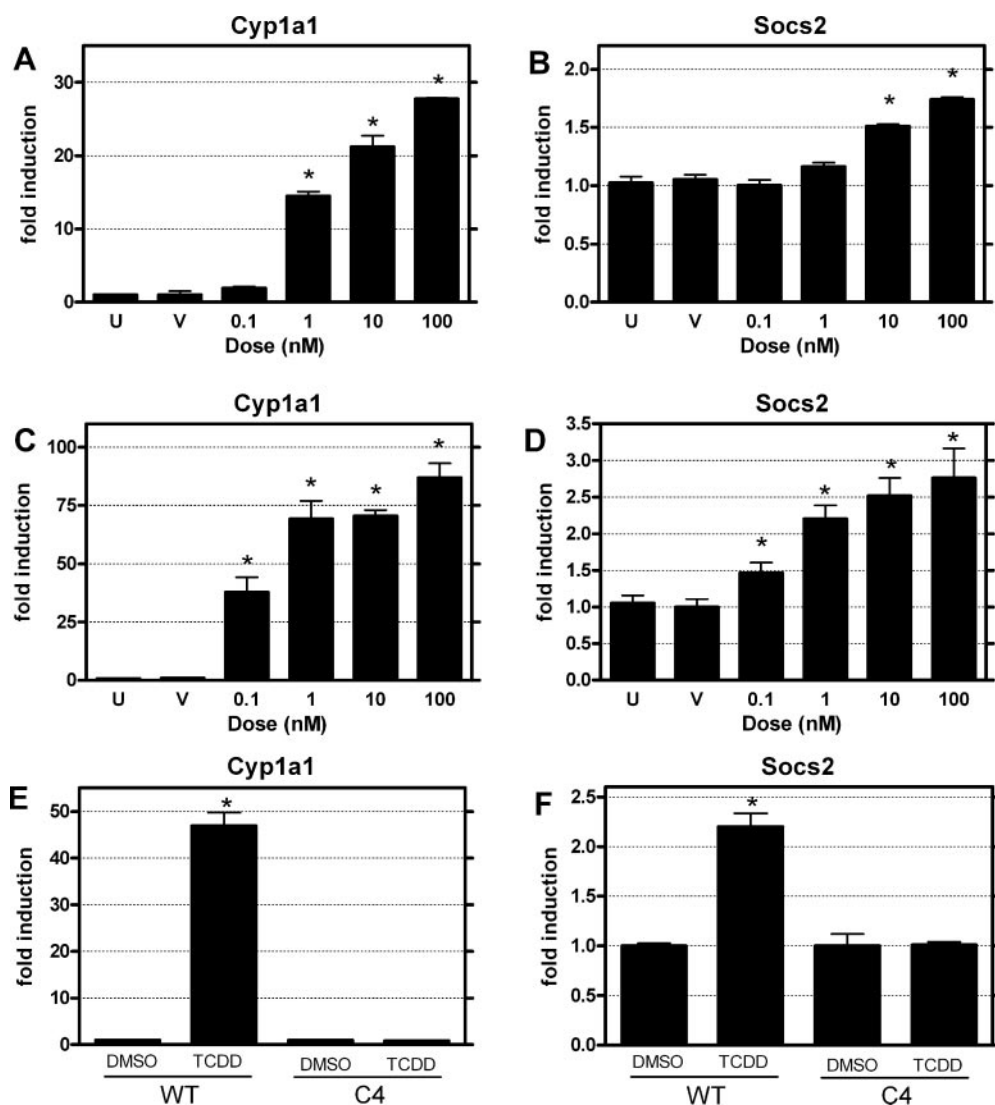


Fig. 6. The Socs2 promoter is activated in response to TCDD in both CH12.LX and Hepa1c1c7 cells and is dependent on the AhR/ARNT signaling pathway. CH12.LX or wild-type Hepa1c1c7 cells were transfected with Cyp1a1 (A and C) or Socs2 (B and D) luciferase reporter constructs and were treated with increasing concentrations of TCDD for 6 h. Luciferase activity was assayed, and induction is reported relative to time-matched vehicle controls. To determine the dependence of the luciferase induction on a functional AhR signaling pathway, Hepa1c1c7 wild-type (WT) and mutant C4 cells (ARNT-defective) were transfected with Cyp1a1 (E) or Socs2 (F) reporter genes and treated with TCDD (10 nM) for 6 h. Induction of luciferase activity was assayed and is expressed as fold induction relative to time-matched vehicle controls. Error bars represent the S.E.M. for the average fold change of at least three independent replicates. *, values significantly different ($p < 0.05$) from the vehicle control. U, untreated control; V, vehicle control.

with the Socs2 promoter, thereby altering its activity in response to TCDD. On the other hand there may be cell differences in chromatin structure that could alter promoter accessibility, and the resultant regulation of transcription. Cyp1a1 mRNA expression patterns also differed in these cell lines, with the induction in CH12.LX cells decreasing rapidly after 4 h, whereas induction in Hepa1c1c7 and MCF-7 cells was sustained. These results indicate additional cell-specific responses to TCDD, which may be mediated by the induction of an AhR transcriptional repressor, a more pronounced activation of ubiquitin-mediated protein degradation of the AhR, or a decreased stability of Cyp1a1 mRNA in CH12.LX cells. Others have noted previously that many gene expression changes mediated by TCDD are cell-line-specific, which indicates the need for caution when extrapolating gene responses and mechanisms of action between tissue/cell lines and across species (Martinez et al., 2002).

Transient transfection assays revealed that the Socs2 reporter gene was activated in response to TCDD in CH12.LX cells. It is surprising that the reporter gene was also activated in the Hepa1c1c7 cells despite the fact that Socs2 mRNA was not induced in this cell line. Similar results have been reported for the human CYP1B1 gene, whose promoter showed TCDD-inducible luciferase activity in HepG2 cells despite the fact that native transcript levels were not induced in response to TCDD (Shehin et al., 2000). Others have suggested that discrepancies between reporter constructs and native gene expression are likely to be caused by factors related to chromatin structure (Eltom et al., 1999). To influence cellular gene expression, activated transcription factors such as the AhR/ARNT heterodimer must be able to access their recognition sites, a process that can be influenced by the presence of nucleosomes and other transcription factors. Transiently transfected reporter genes are devoid of nucleosomes, thereby removing a level of regulation and leaving the promoter sequence completely accessible to AhR/ARNT binding and transcriptional activation. Therefore, the divergent results observed between native gene expression and reporter gene activity in the Hepa1c1c7 cells may indicate that chromatin structure plays a critical role in the transcriptional regulation of Socs2 in these cells.

The induction of Socs2 by TCDD in murine B cells is likely to have important implications in TCDD-mediated immune suppression. Socs proteins are involved in the negative regulation of various physiological signaling pathways, including those of the immune system (Alexander, 2002). Their induction results in the inhibition of signaling cascades such as the Janus tyrosine kinase/signal transducer and activator of transcription pathway, which results in the attenuation of signaling via a classic negative-feedback mechanism. More specifically, Socs2 has been shown to be involved in inhibiting growth hormone, leukemia inhibitory factor, and IL-6 signaling and interacts with the insulin-like growth factor-1 and erythropoietin receptors (Minamoto et al., 1997; Dey et al., 1998; Nicholson et al., 1999; Eyckerman et al., 2001; Greenhalgh et al., 2002). IL-6 is an important cytokine for the terminal differentiation of B cells into antibody-secreting cells (Hirano et al., 1985), and IL-6 null mice exhibit impaired B cell proliferation and antibody production in response to antigen (La Flamme and Pearce, 1999). Therefore, the induction of Socs2 by TCDD may contribute significantly to depressed humoral immunity by inhibiting IL-6 signaling

required for normal B cell proliferation, differentiation, and antibody production. Socs2 knockout mice exhibit gigantism and additional characteristics indicative of deregulated insulin-like growth factor-1 and growth hormone signaling (Metcalf et al., 2000), and Socs2 has also been shown to play an important role in neural differentiation and myeloid leukemia (Schultheis et al., 2002; Turnley et al., 2002). From these effects, it is tempting to speculate on the potential role of Socs2 in TCDD-mediated wasting syndrome and teratogenicity; however, this should be approached with caution before determining its regulation in these tissues.

In summary, the present study has identified and characterized the induction of Socs2 by TCDD in murine B cells. A wide variety of endogenous compounds have been shown to induce Socs2, including various cytokines and hormones (Alexander, 2002; Leung et al., 2003); however, this is the first report of xenobiotic-mediated Socs2 regulation. The profile of Socs2 induction closely parallels that of Cyp1a1, is responsive to diverse AhR-ligands, and is AhR-dependent. The response is cell-line-specific with no induction observed in mouse hepatoma cells or human breast cancer cells. Furthermore, transient transfection studies using both CH12.LX and Hepa1c1c7 cells show that the Socs2 promoter is activated in response to TCDD. It is interesting that TCDD treatment did not induce Socs2 mRNA levels in either Hepa1c1c7 cells or MCF-7 cells, which may suggest that chromatin structure plays an important role in the transcriptional regulation of this gene. Because Socs2 is known to inhibit the signaling of various cytokines and hormones, its induction by TCDD in murine B cells may play a key role in the immune suppression produced by this compound.

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