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Inhibition of the MEK-1/p42 MAP kinase reduces aryl hydrocarbon receptor–DNA interactions

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces expression of the cytochrome P450 1A1 gene, *cyp1a1*, by binding to its receptor, aryl hydrocarbon receptor (AhR). TCDD-bound AhR translocates to the nucleus and forms a heterodimer with its partner protein, AhR nuclear translocator (Arnt). The AhR/Arnt heterodimer then binds to the dioxin-response elements (DREs) in the *cyp1a1* enhancer and stimulates transcription of *cyp1a1*. We tested whether kinase pathways are involved in this process by treating Hepa1c1c7 cells with kinase inhibitors. The MEK-1 inhibitor PD98059 reduced TCDD-induced transcription of *cyp1a1*. TCDD treatment results in phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK), a substrate of MEK-1. Overexpression of dominant negative form of p42 MAPK suppressed TCDD-dependent transcription of a reporter gene controlled by dioxin-response elements (DREs), and pretreatment with PD98059 also blocked this transcription. PD98059 pretreatment also inhibited TCDD-induced DRE binding of the AhR/Arnt heterodimer. Together these results indicate that TCDD activates the MEK-1/p44/p42 MAPK pathway, which in turn activates AhR and so facilitates binding of AhR to the *cyp1a1* DRE.

Keywords: Dioxin; AhR; Arnt; CYP1A1; MAP Kinase; PD98059

Aryl hydrocarbons are environmental contaminants that accumulate in tissues and pose potential risks to human health. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most toxic aryl hydrocarbons and causes numerous abnormalities in experimental animals and humans, including hepatic, pulmonary, and skin tumors in adult rodents, craniofacial deformity during mouse embryogenesis, chloracne, reproductive abnormalities, and immunotoxicity [1–7]. Transcriptional induction of the *cyp1a1* gene encoding cytochrome P-4501A1, a microsomal enzyme that oxygenates aryl hydrocarbons as part of a detoxification process [5,8], represents an adaptive response to TCDD exposure. Upregulation of *cyp1a1* involves AhR, an intracellular TCDD-binding receptor that is a transactivator of *cyp1a1*. Before it binds to its ligand, AhR is found in the cytosol complexed to heat shock protein 90, co-chaperone p23 and Hepatitis B virus X-associated protein 2 [9,10]. Once bound, however, it translocates to the nucleus and forms a heterodimer with the nuclear protein, AhR nuclear translocator (Arnt), and the AhR/Arnt heterodimer recognizes the dioxin-response elements (DREs) in the enhancer region of *cyp1a1*. Dimerization and binding to DRE are both mediated by the basic helix–loop–helix and PAS (bHLH-PAS) domains in the N-terminal halves of both AhR and Arnt [8]. The binding of the AhR/Arnt complex to the DRE increases the transcription of *cyp1a1* by recruiting several proteins that act as coactivators for the formation of the core transcription initiation complex. Beischlag et al. [11] showed that SRC family members such as SRC-1, NcoA-2, and p/CIP are capa-

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ble of enhancing TCDD-inducible gene expression, and that SRC-1 interacts with the helix2 domain but not the C-terminal region of Arnt, whereas the transactivation domain of AhR interacts with all SRC family members. Thus, the SRC family of coactivators is involved in AhR/Arnt-dependent gene expression.

Several recent studies provide insight into the regulation of AhR. They show that AhR is activated by phosphorylation prior to binding to the DRE, since DNA-binding is abolished by phosphatase treatment of AhR in vitro [12]. Moreover, a tyrosine kinase inhibitor reduced dioxin-induced *cyp1a1* gene expression in human keratinocytes [13]. Another study showed that protein kinase C is involved in the nuclear events that result in the binding of AhR to DRE [14].

It has been suggested that other effects of TCDD and AhR also involve protein kinase pathways. For example, TCDD-induced apoptosis of human leukemic T cells is inhibited by pretreatment with tyrosine kinase inhibitors or c-Jun N-terminal kinase inhibitors [3]. There is also evidence that AhR affects cell growth and differentiation by modulating protein kinase pathways. AhR protein levels decrease during adipogenesis, and overexpression of AhR inhibits this process independently of TCDD [15–17]. Hence, AhR may interfere with the cell-cycle arrest required for differentiation.

In this work we investigated the protein kinase pathways involved in the activation of AhR using protein kinase inhibitors. We also performed a number of experiments to determine which step of AhR activation in TCDD-induced *cyp1a1* expression is regulated by the MEK-1/MAPK pathway.

Materials and methods

Cell culture, plasmids, and treatments. Wild type mouse Hepa1c1c7 cells were cultured in α-MEM supplemented with 10% fetal bovine serum (Life Technologies Gibco-BRL) and gentamicin (5 μg/ml) (Life Technologies Gibco-BRL), fungizone (0.25 μg/ml) (Life Technologies Gibco-BRL) under humidified air containing 5% CO_2 at 37 °C. Hepa1c1c7 cells were serum-starved in α-MEM containing 0.5% fetal bovine serum for 40–48 h before treatment with inhibitors or TCDD. Hepa1c1c7 cells were pretreated with 100 μM PD98059 (New England BioLabs) in dimethyl sulfoxide (DMSO). Anti-Arnt antibody was obtained from Transduction Laboratories and anti-AhR antibody was from BIOMOL Research Labs. Anti-phosphorylated p42/p44 MAPK antibody and anti p42/p44 MAPK were obtained from New England BioLabs. Anti-phospho-Serine/Threonine antibody was obtained from Upstate, NY. The pGud-luc1.1 reporter plasmid was provided by Dr. M.S. Denison (University of California, Davis). It contains the firefly luciferase gene and a 484-bp fragment of the upstream region of *cyp1a1* (containing four DRE) that lies directly upstream of the MMTV viral promoter and confers dioxin-responsiveness upon the promoter [18]. pERK1KR and pERK2KR encode dominant negative mutants of p44 (K71R) and p42 (K52R) MAP kinase, respectively [19]. Purified p42 MAPK was obtained from New England BioLabs, [^{35}S]methionine-labeled AhR and Arnt proteins were synthesized by in vitro transcription and translation according to the manufacturer's instructions (Promega).

Transient transfection and luciferase assay. Cells were plated at 1×10^5 cells per well in 12-well plates. Eighteen hours later they were transfected with the DRE-driven reporter plasmid (pGud-luc1.1) together with pCHO110 encoding β-galactosidase, using Lipofectamine-plus reagent according to the manufacturer's instructions (Life Technologies Gibco-BRL). Forty-eight hours after transfection, cell extracts were prepared and analyzed with a luminometer (Berthold Lumat LB9501) using the Luciferase Assay System (Promega). Transfection efficiencies were monitored by measuring β-galactosidase activity as described. Activities were normalized for total protein concentration measured by the Bradford assay (Bio-Rad Laboratories) using bovine serum albumin as standard.

Western and Northern analyses. Western analysis was performed as described [20]. To detect AhR and Arnt, Hepa1c1c7 cells were not serum-starved but grown to 80% confluence in 100-mm tissue culture plates. Total RNA was isolated with RNeasy spin columns according to the manufacturer's instructions (Qiagen). Equal amounts of total RNA were separated by electrophoresis on a 1% agarose gel containing formaldehyde and transferred to a Nytran filter. Blots were hybridized with α-^{32}P-labeled *cyp1a1* cDNA, washed, dried, and analyzed by autoradiography with Hyperfilm MP (Amersham–Pharmacia Biotech) as described. cDNA expression was measured with a phosphoimager (Model Bas2000, Fuji, Japan), and the intensities of protein bands on Western blots were measured with a luminescent image analyzer (Model Las3000, Fuji, Japan).

Preparation of nuclear extracts. Hepa1c1c7 cells were grown to 80% confluence in 100-mm tissue culture plates and treated with inhibitors and TCDD. Cells were washed once with cold 10 mM Hepes [pH 7.5], incubated on ice with cold 10 mM Hepes [pH 7.5] for 15 min, and aspirated. MDH buffer (3 mM $MgCl_2$, 1 mM DTT, 25 mM Hepes [pH 7.5], 0.4 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) was added, and the cells were homogenized by 15 strokes with a Dounce type-B pestle. Nuclei were isolated by centrifuging at 1000*g* for 5 min at 4 °C and washed twice with 1 ml of MDHK buffer (3 mM $MgCl_2$, 1 mM DTT, 25 mM Hepes [pH 7.5], 100 mM KCl, 0.4 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). The isolated nuclei were suspended in HDK buffer (25 mM Hepes [pH 7.5], 1 mM DTT, 400 mM KCl, 0.4 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin), and the suspension was incubated with gentle rocking at 4 °C for 1 h. Nuclear protein was isolated by centrifuging at 15,000 rpm for 1 h at 4 °C [21]. Protein concentrations were measured by the Bradford assay (Bio-Rad Laboratories).

Electrophoretic mobility shift assay (EMSA). Oligonucleotides for DRE (sense: 5′-aattCCAGGCTCTTCTCACGCAACTCCGGGGC-3′, antisense: 5′-aattGCCCCGGAGTTGCGTGAGAAGAGCCTGG-3′) were annealed and labeled (1.75 pmol) with [α-^{32}P]dATP and Klenow enzyme. The DRE sequence represents the dioxin-response element corresponding to nucleotides −1026 to −999 of the promoter/upstream element of rat *cyp1a1* [21]. Nuclear extracts were preincubated with poly(dI·dC) (1.7 μg) in 20 μl HEDG buffer (25 mM Hepes [pH 7.5], 1 mM EDTA, 1 mM DTT, and 10% glycerol) for 15 min at room temperature. The labeled DRE (4×10^5 cpm) was incubated with nuclear extract (10 μg) for 15 min at room temperature and reaction mixtures were separated on 5% PAGE at room temperature. For supershift assays, AhR or Arnt antibody (1 μg) was added for 2 h at 4 °C prior to introducing the labeled DRE.

Whole cell extract and immunoprecipitation. Hepa1c1c7 cells were grown to 80% confluence on 100-mm tissue culture plates and treated with TCDD. The whole cell extracts were prepared as previously described [20]. For immunoprecipitation, 350 μg of whole cell lysates was pre-cleared by incubating with 1 μg of anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and 20 μl of 0.5% ImmunoPure immobilized protein A/G gel (Pierce, Rockford, IL) for 30 min at 4 °C. The cleared extracts were mixed with 3 μg of anti-phospho-Serine/Threonine antibody (Upstate, NY) or anti-AhR antibody (BIOMOL Research Lab., PA). After addition of 30 μl of 0.5% ImmunoPure

immobilized protein A/G gel, mixtures were rotated for overnight at 4 °C. Immunoprecipitated pellets were washed four times with phosphate-buffered saline buffer and then resuspended in SDS sample buffer. The entire samples were boiled for 3 min prior to electrophoresis through an 8% SDS–polyacrylamide gel. The proteins were transferred onto nitrocellulose membrane by semi-dry transfer (Trans-Blot SD; Bio-Rad, Hercules, CA). Co-immunoprecipitated proteins were immunoblotted with anti-AhR antibody and/or anti-phospho-Serine/Threonine antibody. Proteins were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL) with an anti-mouse or rabbit Ig conjugated with horseradish peroxidase (HRP) as a secondary antibody.

Results

Effect of a MEK-1 inhibitor on TCDD-induced expression of cyp1a1

To investigate the possibility that the MEK-1/p42/p44 MAP kinase pathway modulates TCDD-induced gene expression, we measured TCDD-induced expression of *cyp1a1* in Hepa1c1c7 cells that had been pretreated with MEK-1 specific inhibitor, PD98059. Northern analysis showed that pretreatment with the MEK-1 inhibitor PD98059 reduced expression of TCDD-induced expression of *cyp1a1* gene (see Fig. 1).

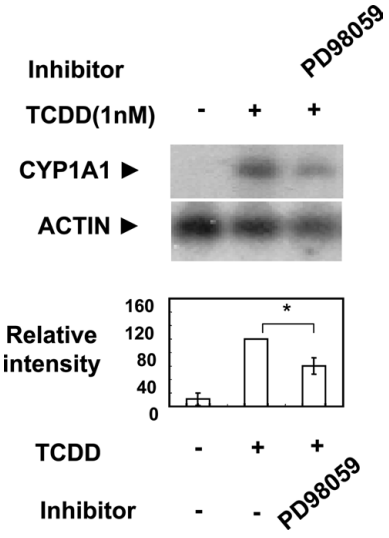

Fig. 1. Effect of PD98059 on dioxin-inducible gene expression Hepa1c1c7 cells were exposed to PD98059 (100 μM) 1 h prior to TCDD (1 nM, 16 h) treatment. After 16 h incubation with TCDD, total RNA was isolated, and 34 μg aliquots were separated on a 1% formaldehyde-containing agarose gel, transferred to a Nytran filter, and hybridized with ^{32}P-labeled CYP1A1 cDNA. As a control, β-actin mRNA levels were measured by hybridization with ^{32}P-labeled β-actin cDNA. Expression of *cyp1a1* was quantitated with a phosphoimager and normalized to the expression of β-actin. Values represent averages and standard deviations of relative levels of *cyp1a1* expression induced by TCDD treatment. *Cyp1a1* expression in TCDD-treated cells was set at 100%. Three separate experiments were performed. The *p* value between TCDD treated sample and TCDD and PD98059 treated sample were calculated by using paired *t* test (*$p < 0.01$).

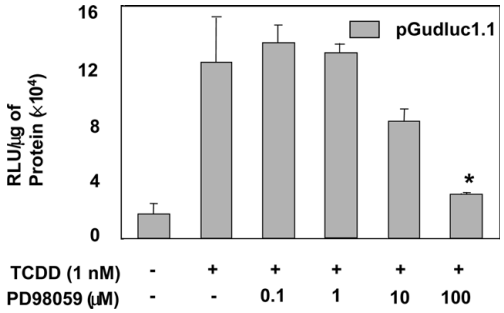

Fig. 2. Effect of PD98059 on DRE-dependent gene expression Hepa1c1c7 cells were transfected with pGud-luc1.1 and treated with various doses of PD98059 for 1 h before TCDD (1 nM) was applied for 16 h. Cell extracts were prepared 48 h after transfection, and luciferase activities were analyzed with a reporter gene assay system (Promega) and normalized by the total protein in each extract. Transfection efficiencies were monitored by measuring β-galactosidase activities. Values shown are means and standard deviations (SD) of three experiments. The *p* value between TCDD treated sample and TCDD plus PD98059 (100 μM) treated sample was calculated by using paired *t* test (*$p < 0.05$).

To test whether PD98059 affects the enhancer activity of *cyp1a1*, we transfected Hepa1c1c7 cells with the reporter plasmid pGud luc1.1 [18]. This reporter encodes luciferase under the control of a 484-bp fragment of the enhancer region of *cyp1a1* that contains four DREs. Because of the presence of these DREs, treatment of the transfectants with TCDD results in luciferase expression (Fig. 2). When the cells were pretreated with PD98059, this expression was inhibited (Fig. 2).

p44/p42 MAPK is necessary for DRE-dependent gene expression

p44/p42 MAPK is a protein kinase that acts downstream of MEK-1. To investigate whether TCDD treatment activates this kinase, Hepa1c1c7 cells were exposed to various doses of TCDD and SDS–PAGE-fractionated whole cell lysates were immunoblotted either with antibody against the tyrosine-phosphorylated form of p44/p42 MAPK or with one that recognizes both phospho- and dephospho-p44/p42 MAPK. Since phosphorylation of p44/p42MAPK is a clear indicator of its activation, we used the anti-phospho-p44/p42 MAPK antibody to evaluate the activity of p44/p42 MAPK. As a positive control for p44/p42 MAPK activation, we also treated the Hepa1c1c7 cells with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) plus ionomycin for 10 min, and found that this strongly activated p44/p42 MAPK. Two hours of TCDD exposure increased the phosphorylation of p44/p42MAPK, and maximal phosphorylation was observed with 1 nM TCDD. Neither TCDD nor TPA altered the level of p44/p42 MAPK (Fig. 3A). Pretreatment of PD98059 suppressed TCDD-induced phosphorylation of p44/p42MAPK (Fig. 3B).

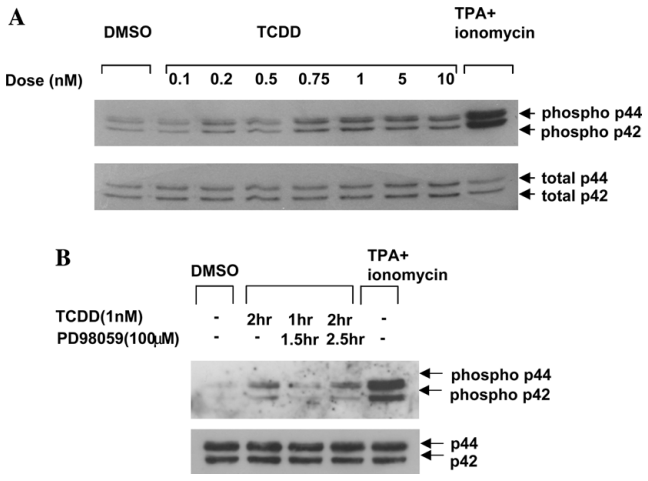

Fig. 3. Effect of TCDD on phosphorylation of p44/p42 MAPK. Hepa1c1c7 cells at 70% confluence were treated with the indicated doses of TCDD for 2 h, or with 10 ng/ml TPA plus 0.5 μM ionomycin for 10 min. Whole cell lysates (70 μg) were fractionated by 10% SDS–PAGE and transferred onto a Nytran filter. (A) The filters were first immunoblotted with antibody specific for the phosphorylated form of p44/42 MAPK (upper panel) and then with general p44/42 MAPK antibody (lower panel). (B) Hepa1c1c7 cells were pretreated PD98059 (100 μM) 30 min prior to the exposure to 1 nM TCDD for indicated hours. The levels of phosphorylated form of p44/42 MAPK (upper panel), and general p44/42 MAPK (lower panel) were visualized by immunoblotting with the corresponding antibodies.

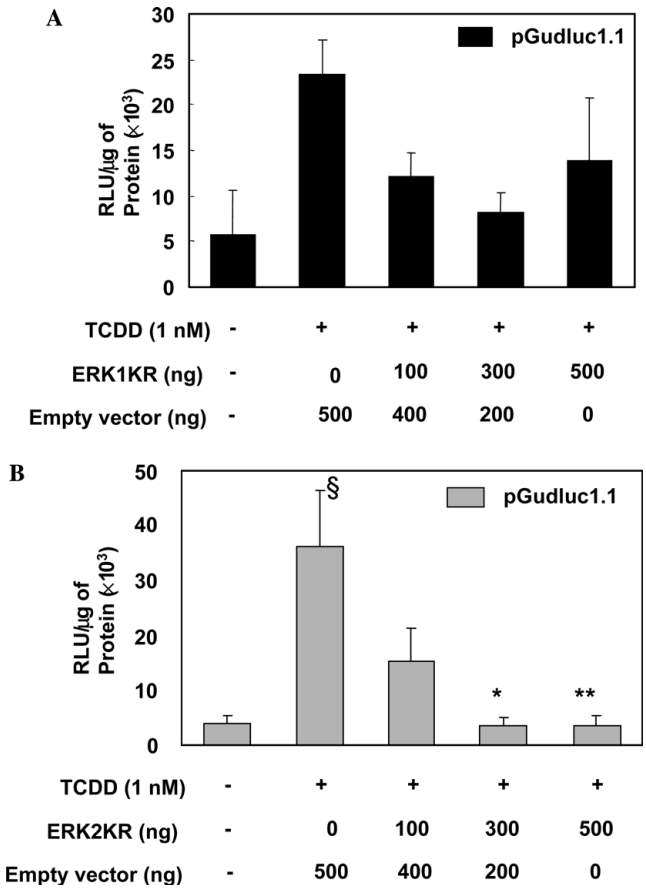

Fig. 4. Effect of dominant negative mutants of p44 and p42 on DRE-dependent gene expression. Hepa1c1c7 cells were transfected with pGud-luc1.1 with or without the indicated amount of plasmids encoding dominant negative mutants (A) p44 MAPK (ERK1KR) or (B) p42 MAPK (ERK2KR). Transfection with empty pcDNA 3.1 vector was included as a control. The cells were treated with 1 nM TCDD or DMSO for 16 h before being harvested. Cell extracts were prepared and luciferase activities were analyzed and normalized by the total protein in each extract. Transfection efficiencies were monitored by measuring β-galactosidase activities. Values shown are means and standard deviations (SD) of three experiments. The p values between control sample (§) and each sample was calculated by using paired t test (*$p < 0.05$; **$p < 0.05$).

To test whether p44/p42 MAPK actually participates in the dioxin-induced transcription of genes that are under the control of DREs, we cotransfected Hepa1c1c7 cells with the DRE-driven reporter plasmid pGud luc 1.1, together with increasing amounts of plasmids that encode dominant negative forms of p44 and p42 (pERK1KR and pERK2KR, respectively) [19]. Cotransfection with pERK2KR dramatically reduced dioxin-dependent expression of the DRE-driven reporter gene (Fig. 4B), and pERK1KR had less pronounced effect. Thus, inhibiting the components of p44/p42 MAPK, particularly p42 MAPK, abrogates dioxin-induced gene activation, thus demonstrating that p42 MAPK is required for dioxin-dependent transcription.

Effect of PD98059 on nuclear translocation

Binding of AhR by TCDD causes translocation of AhR from the cytosol to the nucleus [8], and it has been proposed that AhR undergoes ligand-induced degradation in the cytoplasm following its re-export from the nucleus [22,23]. To determine whether PD98059 affects translocation and ligand-induced degradation, we measured levels of AhR protein in nuclear and cytosolic fractions of PD98059-pretreated Hepa1c1c7 cells exposed to TCDD. PD98059 did not alter either TCDD-induced nuclear localization of AhR or its amount, and PD98059 on its own did not affect AhR localization (Fig. 5, lanes 3 and 8). In addition, nuclear Arnt protein levels were uninfluenced by PD98059 (Fig. 5).

Effect of PD98059 on dioxin-induced DNA binding of AhR/Arnt complexes

To test whether PD98059 blocks binding of AhR to DRE, we performed EMSA. Nuclear extracts of Hepa1c1c7 cells that had been treated with TCDD were mixed with radiolabeled oligonucleotides bearing the DRE sequence of the 5′ enhancer region of *cyp1a1*, and subjected to EMSA [21]. TCDD treatment led to the formation of DRE-binding complexes (Fig. 6A). To examine the composition of these complexes, the nuclear extracts were preincubated with antibodies against

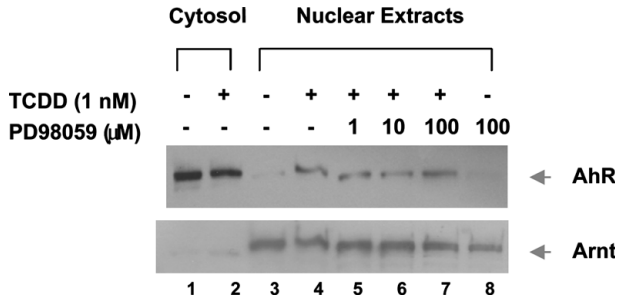

Fig. 5. Effect of PD98059 on nuclear translocation and mobility of AhR. Effect of PD98059 on nuclear and cytosolic levels of AhR and Arnt. Hepa1c1c7 cells were pretreated for 1 h with various concentrations of PD98059, TCDD (1 nM) was applied for 16 h, and cytosolic and nuclear extracts were isolated. Equal amounts of proteins from cytosolic and nuclear extracts (10 μg) were resolved by 8% SDS–PAGE, transferred to a nitrocellulose membrane, and blocked. Thereafter, the blots were probed with anti-AhR antibody for 1 h at room temperature and washed. Bound antibody was visualized by chemiluminescence. The same blot was then stripped, labeled with anti-Arnt antibody, and assessed by chemiluminescence. The results were confirmed by repeated experiments.

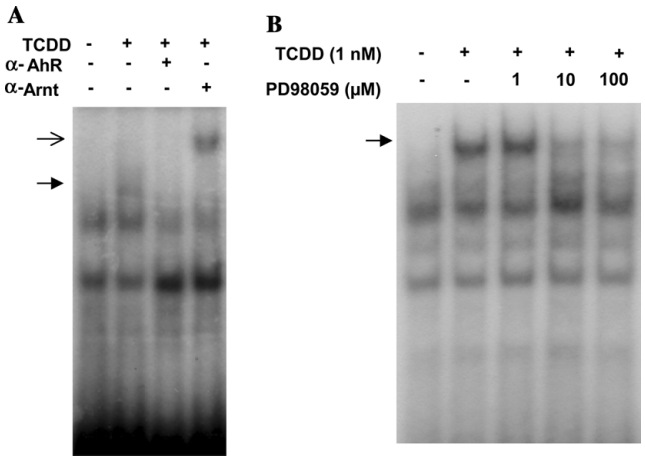

Fig. 6. Effect of PD98059 on TCDD-induced DRE binding by AhR/Arnt complexes. (A) Hepa1c1c7 cells were incubated for 2 h with TCDD (1 nM). Nuclear extracts were isolated and mixed with ^{32}P-labeled oligonucleotides containing a DRE of rat *cyp1a1* (−1026 to −999 of the enhancer element). In some experiments, the extracts were mixed with antibodies specific for AhR or Arnt (1 μg) for 1 h, followed by incubation with radiolabeled oligonucleotides. These mixtures were then analyzed by EMSA. (B) Hepa1c1c7 cells were pretreated with PD98059 for 1 h prior to TCDD treatment (1 nM) for 2 h. Nuclear extracts were then analyzed by EMSA as described in A. Results were confirmed by repeated experiments.

AhR or Arnt. The AhR antibody abolished TCDD-induced complex formation (Fig. 6A, lane 2) and the anti-Arnt antibody supershifted the complexes (Fig. 6A, lane 3). Thus, the TCDD-induced DRE-binding complexes contain AhR and Arnt.

Pretreatment of cells with PD98059 decreased TCDD-induced DNA binding of AhR/Arnt complexes (Figs. 6B) in agreement with the results of the DRE-driven reporter assays (Fig. 2). Thus, PD98059, a MEK-1 inhibitor, specifically impedes the TCDD-induced interaction between DRE elements and AhR/Arnt. Clearly the MEK-1/p44/p42MAPK pathway is implicated in the TCDD-induced interaction between DRE and AhR/Arnt that controls TCDD-induced gene expression.

Serine- or threonine-phosphorylation and AhR

Our results suggest that TCDD induces the activation of p42/p44 MAPK, which affects the DNA-binding activity of AhR. In order to test whether TCDD-activated p42/p44 MAPK directly phosphorylates serine or threonine residues of AhR, we immunoprecipitated cell lysate by using anti-phosphoserine and phosphothreonine antibody and detected co-precipitated AhR by Western analysis using anti-AhR antibody. Our result in Fig. 7A at least suggests that AhR is a component of a protein complex containing phosphoserine or phosphothreonine residues. The amount of co-precipitated AhR is increased by the treatment of TCDD. To test whether AhR itself is phosphorylated, we immunoprecipitated cell lysate using anti-AhR antibody first and detected if there were any phosphoserine or phosphothreonine residues of AhR by Western analysis using anti-phosphoserine and phosphothreonine antibody. The results in Fig. 7B showed that anti-phosphoserine and phosphothreonine antibody fails to recognize AhR. These results indicated that any serine or threonine

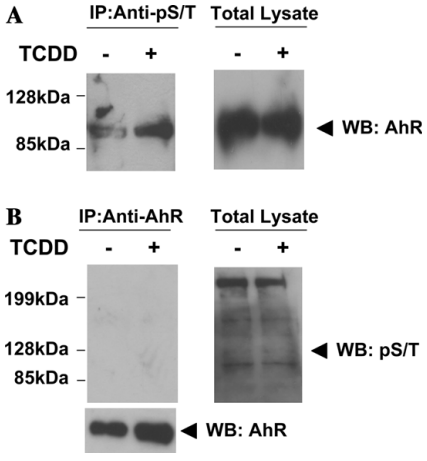

Fig. 7. Interaction between AhR and phosphor-serine/threonine containing proteins. Hepa1c1c7 cells with 80% confluence were treated with TCDD for 4 h. (A) Three hundred and fifty micrograms of whole cell lysates was immunoprecipitated (IP) with anti-phospho-Serine/Threonine antibody (anti-pS/T) for overnight. The resulting immunocomplex (left panel) or total lysate (4 μg) (right panel) was analyzed using anti-AhR antibody by Western blot (WB) analysis. (B) Three hundred and fifty micrograms of whole cell lysates was immunoprecipitated (IP) with anti-AhR antibody for overnight. The resulting immunocomplex (left panel) or total lysate (4 μg) (right panel) was analyzed using anti-phospho-Serine/Threonine antibody (upper). The membrane was reprobed with anti-AhR antibody (lower panel). Results were confirmed by repeated experiments.

residues of AhR are not directly phosphorylated by TCDD. TCDD increases the interaction between AhR and other phosphoproteins.

Discussion

TCDD induces the expression of *cyp1a1*. We showed here that pretreatment of mouse Hepa1c1c7 cells with the MEK-1-specific inhibitor PD98059 reduces this TCDD-induced *cyp1a1* expression. We also observed that TCDD treatment results in the phosphorylation and activation of p42/p44 MAPK, a substrate of MEK-1. Separate experiments indicated that p42 MAPK participates in TCDD-induced *cyp1a1* expression, since blocking p42 MAPK with a dominant negative form of the enzyme inhibited TCDD-induced expression of a DRE-driven reporter. Together these observations imply that TCDD treatment activates MEK-1, which then activates p42 MAPK, and this in turn promotes the interaction between the AhR/Arnt heterodimer and DRE in the enhancer of *cyp1a1*. This interpretation is supported by the fact that the TCDD-induced expression of a DRE-driven reporter gene is blocked by PD98059 pretreatment, and that nuclear AhR/Arnt complexes in TCDD-treated cells that have been pretreated with PD98059 do not bind oligonucleotides representing the DRE-bearing segment of the *cyp1a1* gene enhancer. Co-immunoprecipitation assay suggests that TCDD increases the binding between AhR and phosphoproteins, but not the phosphorylation of AhR. This result suggests that AhR interacts with a protein that is a substrate of serine/threonine protein kinases, such as p42/p44 MAP kinase. This finding suggests that TCDD-activated p42/p44 MAP kinase phosphorylates its putative target protein that interacts with AhR and modulates DRE-binding activity of AhR.

Besides being a selective inhibitor of MEK-1, PD98059 (2′-amino-3′ methoxyflavone) has other functions. Some flavonoid compounds have potential chemopreventive effects by increasing phase II detoxifying enzymes as well as by decreasing activity of CYP1A1 [24,25]. Kang et al. [24] demonstrated that a flavonoid compound, PD98059, induces glutathione *S*-transferase A2 even in cells stably transfected with dominant negative mutant of MEK-1, suggesting that PD98059 has a capability to induce phase II detoxifying enzymes, regardless of MEK-1 activity. They showed that PD98059 induces nuclear translocation of C/EBPβ and activates C/EBPβ-mediated expression of glutathione *S*-transferase A2. Reiners et al. [26] have suggested that PD98059 represses TCDD-induced *cyp1a1* expression by a different mechanism, namely by competing with TCDD for binding to AhR, and so prevents the latter from binding to the DRE. According to that view, PD98059 would act as an AhR antagonist or partial agonist. In addition to this diverse activity of PD98059, we propose that PD98059 interferes with the MEK-1/p42/p44MAPK pathway involved in the TCDD-induced expression of the DRE-driven reporter gene. This idea is supported by our finding that expression of dominant negative mutant of p42 MAP Kinase effectively blocks TCDD-induced expression of DRE driven reporter gene (Fig. 4).

Further support for our interpretation of the action of PD98059 is the recent demonstration that TCDD activates p42/p44 MAPK and Jun N-terminal kinases, but not p38 MAPK in mouse hepatoma cells [27]. Furthermore, TCDD-induced gene expression is repressed by other MEK-1 inhibitor, U0126 [1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butandiene], which does not have a flavonoid-based chemical structure, and by the expression of a dominant negative mutant of MEK-1. Although the activation of p42/p44MAPK by TCDD did not require AhR, AhR was needed for the TCDD-induced upregulation of *cyp1A1*, indicating that AhR is downstream rather than upstream of p42/p44MAPK [27]. Further support for these observations is a study using inhibitors of serine/threonine-specific protein phosphatases such a calyculin A, cyclosporin A, and okadaic acid [28]. p42/p44MAPK is a serine/threonine kinase and its activity is counterbalanced by serine/threonine-specific protein phosphatases. The presence of any of the protein phosphatase inhibitors stimulated TCDD-induced DRE-driven reporter gene expression without affecting the basal level of expression. Treatment with the protein phosphatase 2B inhibitor, cyclosporin A, also increased the interaction between DRE and the AhR/Arnt heterodimer [28]. Also consistent with this view is the observation that phosphatase treatment of TCDD-activated AhR reduces its DRE-binding ability [12,29]. These observations, together with our own, suggest that phosphorylation plays an important role in DRE binding by the TCDD-activated AhR/Arnt complexes [12,30].

Several lines of evidence support the idea that TCDD upregulates protein kinase activity. Indeed, this may be how it promotes tumor progression. First, in mouse lung tumors induced by *N*-nitrosodimethylamine (NDMA), the level of the membrane-associated tumor suppressor, K-ras p21, is decreased, whereas the level of raf-1 and the extent of activation of the MEK-1/p42/p44 MAPK pathway are increased [31]. When these NDMA tumors were treated with TCDD (5 nmol/kg), there was a 2.4-fold increase in tumor burden, a further decrease in membrane-associated K-ras p21, and an increase in total and membrane-associated raf-1; these observations indicate that TCDD promotes tumor development by contributing to the downregulation of membrane-associated K-ras p21 and stimulation of raf-1 [28,31]. Second, TCDD inhibits apoptosis in response to withdrawal of growth factors in the human mammary

epithelial cell line MCF-10A. It has been suggested that TCDD mimics growth factor functions by increasing tyrosine phosphorylation of a variety of signaling proteins and by stimulating the PI3 kinase/Akt pathway that inhibits apoptosis [32].

It is not clear whether TCDD-induced p44/p42 MAPK activity is involved in toxic effects of TCDD other than *cyp1a1* gene induction. The fact that TCDD can modulate the protein kinase pathways involved in proliferation and apoptosis shows that it can engage one or more protein kinase pathways. This effect may be responsible for the physiological and toxic effects of TCDD, such as tumor promotion and infertility. However, it remains to be elucidated how interactions between TCDD and protein kinase signaling can achieve these effects.

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